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Development of alternative host models to assess virulence potential of *Vibrio parahaemolyticus*

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DEVELOPMENT OF ALTERNATIVE HOST MODELS TO ASSESS VIRULENCE
POTENTIAL OF *VIBRIO PARAHAEMOLYTICUS*

BY

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Bachelor of Science, University of Maine at Orono, 2011

THESIS

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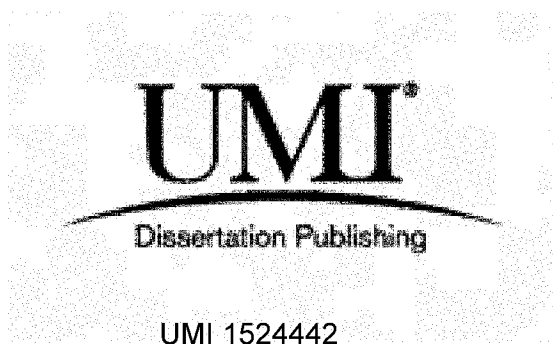
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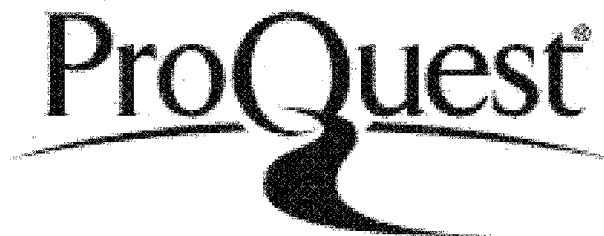
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Abstract

DEVELOPMENT OF ALTERNATIVE HOST MODELS TO ASSESS VIRULENCE POTENTIAL OF *VIBRIO PARAHAEMOLYTICUS*

By

Jeffrey Sun

University of New Hampshire, September, 2013

V. parahaemolyticus infection from ingesting shellfish is a global problem and incidence rates are on the rise in the U.S. Current detection methods are ineffective due to the lack of definite pathogenic marker thus studies to elucidate virulence mechanism are crucial and impeded by the lack of an efficient high-throughput model that emulate disease. Of multiple alternative models investigated, only *C. elegans* was able to discern different virulence potential among *V. parahaemolyticus* strains. In addition, the pandemic strain had greater colonization ability when in direct competition with the pre-pandemic strain in *C. elegans* intestine. A transposon insertion mutant library was generated and screened for changes in virulence-associated traits. Capsule production mutants had decreased virulence and colonization ability in *C. elegans* compared to the wild-type MDOH-04-5M732. *C. elegans* infections were humanized through temperature and the addition of human hormonal cues, thus *C. elegans* is a versatile model for *V. parahaemolyticus* infection studies.

Chapter I

Introduction

Background

The family *Vibrionaceae* is comprised of a diverse group of marine microorganisms found in a wide variety of environmental niches, including in association with hosts. Diverse niche specialization is seen within the *Vibrio* genus, which includes many facultative symbionts that traverse the continuum from mutualistic to pathogenic associations (Thompson et al., 2004). Host association, including very specialized associations, has evolved multiple independent times in this family, suggesting this is a fundamental attribute, although the associations can be very different (Nishiguchi et al., 1992). For example, *Vibrio fischeri* is one of the best-studied *Vibrio* spp. and is known for its mutualistic symbiosis with the Hawaiian bobtail squid *Euprymna scolopes* (Guerrero-Ferreira et al., 2010). To the other extreme, several species including *Vibrio salmonicida*, *Vibrio harveyi*, and *Vibrio coralliilyticus* are pathogenic to marine species including fish, shrimp, and coral (Bjelland et al., 2012; Khemayan et al., 2012; Kimes et al., 2012). Most notorious among *Vibrio* species, however, are those that cause human infections. These include *Vibrio cholerae*, the causative agent of cholera, *Vibrio vulnificus*, the most deadly *Vibrio* in the U.S. responsible for the most fatalities from food poisoning and wound infections (between 30-32% depending on the data source), and *Vibrio parahaemolyticus*, an emergent opportunistic pathogen that the CDC estimated in 2011 caused 56% of all Vibriosis cases within the U.S., whereas data

compiled from the FOODNET in 2012 indicate that *V. parahaemolyticus* caused 54% of all food-borne vibrio infections, mostly from contaminated shellfish (Daniels et al., 2000; Newton et al., 2012; Thompson et al., 2004). There are a number of challenges for pathogens and symbionts to successfully colonize host or survive in distinct niches, such as limited nutrients, salinity changes, and temperature shifts. The acquisition of new traits can aid in successful niche expansion or host specialization (e.g. to new hosts); although some gain traits able to infect a diverse lineage of organisms (Shinoda et al., 2010).

Pathogenic *Vibrios* are naturally found in the marine environment and cause periodic devastating outbreaks, epidemics, and pandemics (Thompson et al., 2004). *V. cholerae* causes severe dysentery through the action of cholera toxin and are spread normally through the oral-fecal route (Cinar et al., 2010). *V. vulnificus* is especially virulent among patients with liver disease and iron storage disorders, who are at increased risk of invasive disease that frequently leads to sepsis and death (Blake et al., 1979). *V. parahaemolyticus* is endemic to the temperate estuarine waters of the United States and infection is associated with the consumption of raw or undercooked seafood (Shimohata et al., 2010). Clinical features most commonly associated with *V. parahaemolyticus* infection include watery diarrhea, abdominal cramps, nausea, and vomiting; with wound infections and septicemia occurring less commonly (Daniels et al., 2000).

Several epidemiological studies in recent years indicate that *V. parahaemolyticus* is an emergent disease of concern within the United States. A review of Cholera and other *Vibrio* infections (COVIS) from 1997 to 2006 identified 4,754 cases of *Vibrio* species-related illnesses, 1,210 were non-foodborne *Vibrio* infections (Deschet et al., 2008). This was a vast difference from *V. vulnificus* which caused 1 outbreak with 459

reported illnesses (Iwamoto et al., 2010). These cases are an underestimation of the actual number of cases for *V. parahaemolyticus* since the infection is self-limiting and many are not reported. In fact, the CDC estimates that for every reported case, 146 cases go unreported (Figure 1.1; <http://www.cdc.gov/features/dsfoodnet2012/>). Due to global climate change and several years of higher than average ocean water temperatures, the rates of infection have increased dramatically worldwide (Hansen et al., 2006). Based on recent data from the 10-state food-borne disease active surveillance network (FOODNET) surveillance, *V. parahaemolyticus* is the most common seafood-born bacterial infection in the United States.

Since 1988, the CDC maintained a voluntary surveillance system for culture-confirmed *Vibrio* infections in the Gulf coast region and this surveillance was expanded to nation-wide in 2007. In 2011, the CDC estimates a total of 45,000 *V. parahaemolyticus* cases yearly in the U.S. (90% confidence interval (CI), 23,000-75,000), 207 cases of *V. vulnificus* (90% CI, 138-287) and 35,000 cases of *Vibrio* species (90% CI, 22,000-52,000; Scallen et al., 2011). Thus, this bacterium is a serious food safety concern for the consumers of seafood. For this reason, research that improves the ability to identify pathogenic *V. parahaemolyticus* is important to predicting and preventing future outbreaks. The CDC currently monitors vibriosis through 2 surveillance systems: COVIS, which conducts passive surveillance and FOODNET that conducts active surveillance for laboratory-confirmed *Vibrio* infections (Newton et al., 2012). Current preventative measures include annual risk assessment of oyster harvesting areas based on a standard protocol involving environmental monitoring (NSSP 2011).

Emergence of disease causing strains of *V. parahaemolyticus*

Prior to 1997, illnesses resulting from the consumption of raw or undercooked shellfish within the U.S. were sporadic. The first confirmed oyster-associated outbreak caused by *V. parahaemolyticus* in the U.S. occurred in the summer of 1997 with oysters harvested in Washington. Recurrent smaller outbreaks occurred in the following year in Washington. The largest outbreak in U.S. history occurred in 1998 from the consumption of oysters from Galveston Bay, TX (DePaola et al., 2000). Recent outbreaks in Massachusetts, New York, New Jersey, Connecticut, and Alaska demonstrate that pathogenic *V. parahaemolyticus* are no longer limited to warmer waters. The largest outbreak in Galveston Bay, TX was distinguished by an extremely high infection rate and by the fact that all the clinical isolates belong to a single clone group (O3:K6 serotype).

V. parahaemolyticus isolates are classified using a serotyping system based on the lipopolysaccharide (O) and capsular (K) antigens. Isolates are diverse with 12 O groups and 65 K groups recognized. Even with the diversity, there are serotypes that have higher prevalence among others. Between 1979 and 1995, the O4:K12 serotype had the highest prevalence among clinical strains tested from the U.S. Pacific Coast. However, a new clone of the O3:K6 serotype emerged in India in 1996 and quickly spread throughout Asia. A year later the isolation of this clone in Peru resulted in a major shift in the population dynamic of *V. parahaemolyticus* in this region. The arrival of this pandemic strain replaced the seasonal and self-limited infections attributed to the native population and led to the appearance of diverse serovariants containing genomic regions characteristic of the pandemic clone not detected previously (Gavilan et al., 2013). This suggests the occurrence of genetic transfer from the introduced population to the local

communities which could lead to an increased incidence of infection by the native population.

Virulence by *V. parahaemolyticus* and obstacles for identifying pathogenic strains in the environment

The majority of environmental *V. parahaemolyticus* strains are innocuous members of the marine microbiota with only small subpopulations being pathogens of humans. These pathogenic subpopulations have the potential to cause periodic outbreaks, epidemics, and pandemics (Turner et al., 2013). Environmental factors, such as temperature, salinity, and rainfall, influence that abundance and distribution of *V. parahaemolyticus* populations. Regulatory authorities of recreational waters and shellfish harvesting areas currently employ surveying rainfall, fecal coliform counts, river stages, and enterococcus counts to determine the opening and closing of specific areas. These approaches are not useful for identifying the presence of the pathogenic subpopulations of *Vibrio* spp. (Johnson et al., 2012). Current research on *V. parahaemolyticus* is driven by the need to detect these pathogens in the environment (Panicker et al., 2004). This would require the ability to discriminate between the pathogenic from the non-pathogenic strains.

The classical markers used in identifying pathogenic *V. parahaemolyticus* strains are the thermostable direct hemolysin (*tdh*) gene encoding TDH and thermostable-related hemolysin (*trh*) genes encoding TRH. The TDH protein produces a number of cytotoxic effects which includes increasing the permeability of human erythrocytes and the secretion of chloride in intestinal cells, disrupting the microtubule cytoskeleton and

epithelial barrier function, and cell rounding (Caburlotto et al., 2010; Pineryo et al 2010). Outbreaks with strains missing the *tdh* and *trh* markers have occurred and a high concentration of bacteria with these genes does not always increase the number of outbreaks (Garcia et al., 2009; Strom et al., 2010). Purified TRH protein induces lysis of erythrocytes and fluid accumulation in the rabbit ileal loop model (Caburlotto et al., 2010). However, *V. parahaemolyticus* strains lacking the *tdh* and *trh* genes can still cause the destruction of the epithelial barrier in the absence of these toxins (Lynch et al., 2005). In addition to the classical virulence markers, a type 3 secretion system 2 (T3SS2) plays a role in virulence. The T3SS2 secretes and translocate virulence factors known as effectors into eukaryotic cells (Caburlotto et al., 2010). These effectors are involved in enterotoxicity in the rabbit ileal loop model as well as cytotoxic activity against CaCo-2 and HCT-8 cells (Hiyoshi et al., 2010). The deletion of the *tdh* gene does not affect cytotoxicity against HeLa cells and results in a partial fluid accumulation in the rabbit ileal loop model. This indicates the existence of other virulence factors that are currently unidentified (Broberg et al., 2011).

Pathogenic *Vibrios* produces other virulence-related factors which are not proven to contribute to disease or are not tested due to the lack of model, but may be important in disease. These include enterotoxins, hemolysin, cytotoxins, proteases, siderophores, adhesive factors, motility, and hemagglutinin (Mahoney et al., 2010; Shimohata et al., 2010). These virulence-related factors may play a role in both survival in the environment as well as colonization of the human host. Temperature regulation plays a role in virulence gene expression for clinical strains of *Vibrio parahaemolyticus* when compared to environmental strains (Mahoney et al., 2010). Expression of known and putative

virulence-associated traits by clinical reference isolates correlated with increased temperature from 28°C to 37°C, as compared to the environmental isolates which did not induce their putative virulence-associated traits in response to a temperature shift to 37°C (Mahoney et al., 2010). Motility, biofilm production and protease production of clinical strains had a significant increase due to the temperature shift. However, the protease production by environmental strains significantly decreased at 37°C relative to 28°C. These proteases produced by pathogenic *Vibrios* play a role in virulence in subsequent infections (Shimohata et al., 2010).

Disease models in use for *V. parahaemolyticus* and their limitations

Currently the characterization of how *V. parahaemolyticus* causes disease has yielded the identification of some virulence factors and toxins. However studying each individual factor will not elucidate the ability of this bacterium to cause disease. This is because *V. parahaemolyticus* must utilize both effectors and toxins to work in concert to cause a disease progression. The ability to study the virulence mechanisms of any pathogen requires the use of laboratory models for infection. For *V. parahaemolyticus*, only a few models have been applied to study such as the infant mouse gastric model, the orogastric piglet model, the rabbit ileal loop model, and cell culture models (Caburlotto et al., 2010; Hiyoshi et al., 2010; Lynch et al., 2005; Nishibuchi et al., 1992; Pineryo et al., 2010). These models have a body temperature that is similar to the human body temperature which is crucial for the expression of temperature regulated virulence (Mahoney et al., 2010). In addition, the use of these vertebrate models is costly, time-consuming, and present regulatory challenges even when justifiable.

The mouse orogastric model preserves the natural route of infection for *V. parahaemolyticus*; however, for many studies bacteria are introduced through peritoneal injections (Hiyoshi et al., 2010; Hoashi et al., 1990). Mice infected through either method have similar pathological changes such as swelling, redness, and fluid accumulation in the small intestine (Hoashi et al., 1990). Mice lethality and pathological findings from *V. parahaemolyticus* infection were irrespective of the hemolysin phenotype of the strain used, thus mice infections may be attributed to other factors besides hemolysin (Hiyoshi et al., 2010; Hoashi et al., 1990). However, mice infection based on the progression of disease is unreliable. In replicate experiments, 4-6 day-old infant mice infected with pathogenic strains of *V. parahaemolyticus* became symptomatic and then recovered, whereas other symptomatic mice succumbed to disease (as determined by loss of palor beyond recovery, at which time mice were euthanized). This unacceptable variability is attributed to the slight differences in development between mice in replicate experiments (Mahoney et al., 2011).

For the rabbit ileal loop model bacteria or purified toxin are injected and fluid accumulation is measured. This model has been successfully applied for characterizing the action of T3SS2 effectors, which are now well established based on this and other work as important for human disease. In the rabbit ileal loop model, intestinal fluid accumulation diminished when infected by mutants containing a deletion of the T3SS3 related gene, *VopP*; whereas, fluid accumulation was unaffected when infected with mutants with deletion in the T3SS1 related genes (Park et al., 2004). The rabbit ileal loop model has also identified the T3SS2 translocon proteins, VopB2 and VopD2, as playing a crucial role in the enterotoxicity of *V. parahaemolyticus* (Kodama et al., 2008). However,

the rabbit ileal loop infections require surgical procedures and high level of expertise, which limits the use of this infection model.

Orogastric pig is an infection model that preserves the natural route of infection of *V. parahaemolyticus*. Piglet model infections can be evaluated for clinical signs of infection, such as diarrhea, as well as pathological findings from dissection. Piglet infection of *V. parahaemolyticus* requires the presence of a functional T3SS2 to induce gastrointestinal disease such as diarrhea and vomiting. However, pig intestines had minimal histopathological abnormalities, such as distention or edema, even when infected with wild-type *V. parahaemolyticus* (Pineryo et al., 2010). The inconsistencies between the clinical signs and histology, in addition to the limitation on the number of animals needed for infection studies, makes the orogastric pig model a less efficient model for *V. parahaemolyticus* infection.

Cell culture of human cells facilitates studies of the intestinal barrier and is used to test the cytotoxicity caused by *V. parahaemolyticus* and is subject to far fewer regulatory hurdles than whole animal vertebrate studies. This pathogen causes cytotoxicity towards human cells which is well established among researchers of pathogenicity to be a measure of virulence (Carbulotta et al., 2010). However, this cytotoxicity is dependent primarily on T3SS1, which is ancestral in the species, and present in all strains, even those that are not pathogenic. Furthermore, the relevance of this attribute and other effects of *V. parahaemolyticus* on cultured cells to human disease is not established. The induction of Caco-2 cell death occurs through the activation of the three major MAPK signaling pathways by *V. parahaemolyticus*. This activation requires the presence of the T3SS1 effector VP1680 (Matlawska-Wasowska et al., 2010). In HeLa cells, the ExsE

protein contributes to the regulation of an adherence phenotype that is required for translocation of T3SS1 effector proteins (Erwin et al., 2012). The use of cell culture allows for the study of cytotoxic effects during *V. parahaemolyticus* infection; however, cytotoxic effects do not equate to virulence; presumably non-pathogenic environmental isolates of *V. parahaemolyticus* from New Hampshire that lack T3SS2, were more cytotoxic than the clinical strains of *V. parahaemolyticus* (Mahoney et al., 2010). Thus the applicability of cell culture for the study of virulence is questionable, and requires careful assessment of non-pathogenic strains as controls, and further requires that any effect observed be evaluated in a more relevant model against non-pathogens.

Alternative models for bacterial pathogenesis and their potential for use with *V. parahaemolyticus*

Alternative invertebrate host models are successful surrogates for infection studies of many pathogens such as *Salmonella typhimurium*, *Pseudomonas aeruginosa*, and *Burkholderia cenocepacia* (Aballey et al., 2001; Cooper et al., 2009; Petersen et al., 2012). Alternative host models, such as *Drosophila melanogaster* (fruit fly), *Caenorabditis elegans* (nematode), and *Manduca sexta* (tobacco hornnematode) allow the study of human pathogens by circumventing the issues faced with using vertebrate models. Fruit flies and nematodes are easily maintained and continuously produce large numbers of progenies once a stock population is obtained. Tobacco hornnematodes larval must be ordered for each experiment due to difficulties in obtaining progenies without specialized growth chambers. These models are beneficial in studying gastrointestinal pathogens since the route of infection can be preserved. Nematodes develop the fastest

with a 3 day life cycle, whereas fruit flies and tobacco hornworms mature after about a week. Infection of these alternative models is normally monitored through death; however, fruit flies and the tobacco hornworm can also be weighed to determine weight loss from symptoms such as diarrhea.

D. melanogaster is an accurate and inexpensive model for the elucidation of host susceptibility to cholera (Blow et al., 2005). Infection experiments utilizing *V. cholerae* mimicked the human disease cholera and helped elucidate some virulence mechanisms. Ingestion of cholera toxin by mammals will result in severe secretory diarrhea and they found that ingestion of purified cholera toxin is not lethal to the flies. However, the cholera toxin is lethal to flies when a pathogenic strain of *V. cholerae* with a deletion of the cholera toxin genes is co-ingested. Even though differences in infection exist between mammals and flies, the hallmarks of human cholera disease is still seen in the flies (Blow et al., 2005). This model is simple to handle, genetically tractable, and has a well-studied innate immunity system. The ease of this model and success with *V. cholerae* infections has encouraged us to investigate this model for *V. parahaemolyticus* infections.

C. elegans is an invertebrate host used to identify and assess virulence factors of the human pathogens *Salmonella typhimurium*, *Yersinia pseudotuberculosis*, *Serratia marcescens*, and *Burkholderia pseudomallei* (Cinar et al., 2010; Dhakal et al., 2006). The use of *C. elegans* as a model organism for over 3 decades is due to its many advantages including simple growth conditions, a short life cycle, and a fixed cell lineage with a full sequenced genome (97 Mb; Aballay et al., 2002; Cinar et al., 2010). There is a wealth of data demonstrating that a variety of developmental, neurological, cell biological, and biochemical processes are highly conserved between nematodes and mammals (Aballay

et al., 2001). Direct competitions in nematodes are possible with fluorescently labeled bacteria due to their translucent physiology. In an effort to evaluate the relevance of nematode infections to mammals and a variety of divergent host, *P. aeruginosa* strain PA14 infections were conducted. This strain is capable of infecting both mice and *Arabidopsis thaliana* (plant) and resulting in symptomatic mice and yellowing of the plant. They found that mutations in a variety of PA14 virulence-related genes resulted in a significantly reduced virulence in mice, nematodes, and plants (Tan et al., 1999).

The nematode model is used in infection studies to investigate the virulence mechanism of the sister genus of *V. parahaemolyticus*. The virulence factors essential to mammalian *V. vulnificus* infections also play important roles in *C. elegans* killing. In this pathogen, mutants missing the global virulence regulator CRP and exotoxin exhibited increased nematode killing time (Dhakal et al., 2006). With *V. cholerae*, it was seen that lethal infections in the nematodes were caused via a cholera toxin and toxin co-regulated pili independent process (Sahu et al., 2012). This would provide a useful host model system to screen other virulence factors crucial to cholera infections.

The invertebrate model *Manduca sexta* (tobacco hornnematode) is a well-known insect model used to elucidate the infection of many human pathogens, such as *Yersinia pestis* and *Serratia marcescens* (Fuchs et al., 2008; Petersen et al., 2012). Tobacco hornnematodes can be infected through either injections or fed orally and disease is normally associated to weight loss or death of the hornnematodes. In addition, the tobacco hornnematodes can adapt to higher temperatures such as the human body temperature to elucidate temperature regulation of virulence in many human pathogens. The expression of putative temperature regulated virulence genes at different

temperatures plays a key role in the virulence of *Serratia marcescens* SCBI in tobacco hornworms (Petersen et al., 2012).

The utilization of these alternative models as surrogates for mammalian infection has led to the identification of many regulatory genes, metabolic processes, and toxins that contribute to pathogen virulence in diverse host lineages. In *P. aeruginosa* studies, the same virulence factors are usually required for a successful infection and host response is more similar (Hendrickson et al., 2001). In that study, 21 genes identified as being involved in pathogenesis by screening transposon-induced PA14 mutants. Utilizing *A. thaliana*, nematodes, wax moths, and mice, it was seen that 18, 17, 19, and 21 of these genes were required for pathogenicity respectively. Even though these models are evolutionarily distant from one another, the fact that the same virulence genes are necessary for successful infection means that some virulence genes are universal and host responses are conserved (Hendrickson et al., 2001).

Virulence gene expression and the growth of bacteria are influenced by many environmental factors such as temperature and molecular cues. Temperature regulation plays a key role in the expression of specific virulence-associated traits such as protease, motility, and biofilm in *V. parahaemolyticus* (Mahoney et al., 2010). These traits have a significant increase in expression based on phenotypic assays when exposed to the human body temperature. The use of *C. elegans* in *V. parahaemolyticus* gastric infection studies will circumvent the issues with the use of invertebrate models. However, as an invertebrate model, the internal body temperature and host hormones are not similar to humans. The human hormone norepinephrine is able to enhance the growth rate and virulence of many gastrointestinal pathogens such as enterohemorrhagic *Escherichia coli*

and *V. parahaemolyticus* (Nakano et al., 2006). In *V. parahaemolyticus*, norepinephrine enhances cytotoxicity in CaCo-2 cells and enterotoxicity in the rabbit ileal loop. Thus, the artificial manipulation of temperature and the addition of norepinephrine during infection assays would allow *C. elegans* infections to emulate normal human conditions to a greater degree and strengthen this alternative model in elucidating the virulence mechanism of *V. parahaemolyticus*.

Objectives, Goals, Hypotheses and Research Questions:

Arguably, the single greatest obstacle for preventing infections by *Vibrio parahaemolyticus* is the lack of understanding of its disease mechanisms. A better knowledge of the genetic determinants of transmission and virulence would facilitate the development of sensitive detection methods. These methods could then be used to determine whether shellfish is safe and could possibly fuel the study of environmental conditions that lead to outbreaks. My research could also inform the development of specific treatment or preventative regimes for at-risk groups. Because some of the approaches used herein are based on comparisons with other *Vibrio* pathogens and informed by the more extensive knowledge base on virulence with those other species (especially *Vibrio cholerae*), these studies will also allow the testing of specific hypotheses about the conservation of disease mechanisms within the genus as attributes of the core genome, as well as examine the contribution of horizontal elements to the evolution of virulence by some strains within this species.

The use of an effective and efficient model for infection that allows the study of disease by *V. parahaemolyticus* is crucial in being able to identify virulence and transmission factors of this pathogen. This improved knowledge will enable better preventative measures in decreasing human infections. The ideal model will differentiate pathogens and non-pathogens and be capable of elucidating subtle virulence differences between pathogens. My research will address both broad and specific questions regarding the virulence of pathogens and will address three specific hypotheses:

Chapter II:

Can *V. parahaemolyticus* cause disease in an alternative (invertebrate) infection model? If so, do some models display disease more accurately? Which models are most consistent, or have the highest potential for high throughput applications?

Hypothesis: The presence of universal host responses during infection allows the use of alternative non-mammalian hosts as surrogates in revealing the *in vivo* virulence mechanism of *V. parahaemolyticus*.

Approach: Compare infection of alternative models using 10 select control bacterial strains to determine bacterial virulence differences. Host colonization by the pandemic, pre-pandemic, attenuated, and environmental *V. parahaemolyticus* strains was determined by culture methods and observed by microscopy. Pair-wise competitions of select strains were performed using fluorescently labeled bacteria. The most efficient model was used in elucidating virulence potential of transposon mutants generated.

Chapter III:

Do traits that are associated with the virulence of other bacterial pathogens contribute to disease by *V. parahaemolyticus*? What genes are crucial for the expression and temperature regulation of virulence-associated traits?

Hypothesis: Virulence associated traits that contribute to disease or transmission by other *Vibrio* species, also contribute to disease by *V. parahaemolyticus*.

Approach: Transposon mutagenesis was applied and mutants assessed for alterations in protease, hemolysin, motility, biofilm, siderophore, capsule and auxotrophy. Several of these assays were performed at both 28°C and 37°C to identify mutants with altered temperature regulation. Select mutants were characterized in the newly developed nematode model, and the identity of disrupted gene determined.

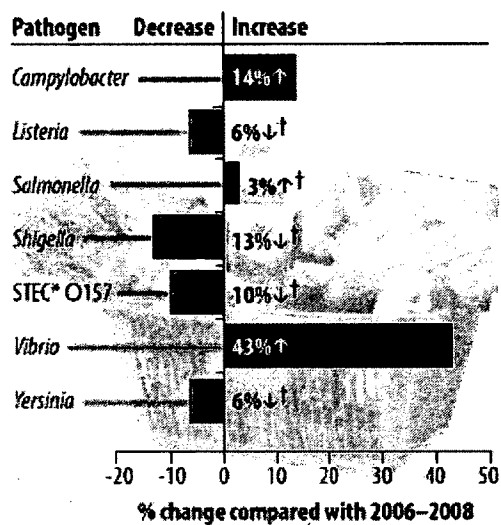
Chapter IV:

Does the presence of norepinephrine enhance virulence of pathogenic *V. parahaemolyticus* strains? Is this virulence enhancement observed in environmental strains?

Hypothesis: Because the presence of norepinephrine enhances the virulence of pathogenic *V. parahaemolyticus* in mammalian models, it will also enhance virulence in *C. elegans* but it will not enhance the virulence of non-pathogenic strains.

Approach: Bacteria were grown in the presence of norepinephrine and fed to the *C. elegans* gastric infection model. Virulence was determined in the nematode infection model by mobility. Phenotypic assays were performed to determine the source of virulence restoration in the GacA⁻ mutant. Confocal microscopy was employed to determine the effect of norepinephrine on the colonization ability of GacA⁻ in competition.

**Changes in incidence of laboratory-confirmed
bacterial infections, US, 2012**



*Shiga toxin-producing *Escherichia coli*

†Not statistically significant

Figure 1.1. Changes in incidence of laboratory-confirmed bacterial infections, United States, 2012 compared with 2006–2008. Courtesy of the CDC.

Chapter II

Comparison of Alternative Models to Assess Virulence Potential of *Vibrio parahaemolyticus*

ABSTRACT:

Current efforts in understanding the virulence of *Vibrio parahaemolyticus* are hindered by the lack of a cost-efficient and dependable model that emulates disease. We assessed the ability of *V. parahaemolyticus* to induce gastric disease in multiple invertebrate models, including the fruit fly *D. melanogaster*, tobacco horn nematode *M. sexta*, and nematode *C. elegans*, all of which have been useful for identifying virulence factors in other pathogens. In addition to known pathogen and non-pathogen treatments used in previous studies published with these models, we included six *V. parahaemolyticus* strains with expected differences in virulence. The fruit fly and tobacco horn nematode displayed highly variable weight change following gastric infection by pathogenic strains of *V. parahaemolyticus*. However, nematodes displayed more consistent responses to gastric infections by human pathogenic *V. parahaemolyticus*. Although these infections were not lethal they did result in a significant reduction in mobility compared to a mutant strain with attenuated virulence, and a presumed non-pathogenic environmental isolate with no known virulence genes. For nematodes exposed to fluorescently labeled wild-type strains, mobility inversely correlated with proficiency

in intestinal colonization, which was most apparent from pair-wise competitions determined both visually and by culture based enumeration.

INTRODUCTION:

Vibrio parahaemolyticus is a naturally occurring and typically harmless marine inhabitant but it is also the leading cause of seafood-borne gastroenteritis worldwide. Infections by rare pathogenic strains normally occur through their ingestion in raw or undercooked seafood; however, this pathogen is also capable of causing wound infections and rarely, life-threatening septicemia (Broberg et al., 2011; Lynch et al., 2005; Pineryo et al., 2010). Clinical features most commonly presented during inflammatory gastroenteritis include watery diarrhea, abdominal cramps, nausea, and vomiting (Daniels et al., 2004). In the United States, measures have been in place for some time to protect consumers of shellfish from the warmer waters of the Gulf region. However, recurrent outbreaks in Washington, and more recent outbreaks in Massachusetts, New York, New Jersey, Connecticut, and Alaska highlight that the pathogen is not limited to warmer climates and emphasizes the importance of understanding the virulence mechanism of this emergent pathogen in order to prevent disease (DePaola et al., 2003; Mahoney et al., 2010; Newton et al., 2012).

A major limitation for the understanding of the virulence of *Vibrio parahaemolyticus* is the lack of a reliable high-throughput disease model allowing the identification and characterization of virulence factors. Mammalian models including orogastric mice, suckling piglets, and rabbit ileal loops as well as cultured human cells

have provided insight into the role of several proposed virulence factors in disease. These virulence factors include the major toxins thermostable direct hemolysin (TDH), *tdh*-related hemolysin (TRH) that are often but not always associated with clinical strains and when deleted do not alter animal virulence, and the type III secretion systems (TTSS1 and TTSS2); thus, these factors alone are not determinants of virulence (Garcia et al., 2009; Hoashi et al., 1990; Hiyoshi et al., 2010; Park et al., 2004; Pineryo et al., 2010). The vertebrate and tissue culture models do emulate human conditions such as temperature which may be crucial for the expression of some virulence-associated traits (Mahoney et al., 2010). Mouse and piglet gastric models preserve the natural route of infection (Pineryo et al., 2010). However, these models each also have shortcomings. Disease progression in the infant mouse gastric model is highly variable between replicates and dependent on mouse developmental (Mahoney et al., 2011); whereas piglet infections do not result in histologies that correlate to disease symptom (Pineryo et al., 2010). The rabbit ileal loop demonstrated the role of T3SS2 effectors (Park et al., 2004; Kodama et al., 2008); however, this model requires a high level of expertise which limits its use. The above vertebrate models are not suitable for high-throughput screening for unknown virulence factors. Use of cultured human cells provides a more high throughput model for virulence often with cytotoxicity as the disease symptom (Erwin et al., 2012; Matlawska-Wasowska et al., 2010), but non-pathogenic *V. parahaemolyticus* strains are more cytotoxic than clinical isolates (Mahoney et al., 2010), calling into question the validity of cytotoxicity as a measurement of virulence.

Alternatively, the use of simpler, inexpensive, and practical surrogate hosts to study this pathogen would provide a way of overcoming some obstacles of vertebrate

models, especially for high-throughput screens. The interaction between a pathogen and the host is a dynamic process and requires the infecting organism to express a variety of virulence factors in order to overcome host defense mechanism to establish an infection. Some of these virulence factors, such as toxins and effectors, may not be host specific and can be utilized for pathogenicity upon diverse hosts. Microbe-host studies have revealed the conservation of innate defense mechanisms among plants, invertebrates, and mammals (Starkey et al., 2009). This suggests that some host infection responses are universal among diverse lineages. Some of the most common alternative models in current use with other pathogens include fruit flies, nematodes, and the tobacco hornworms. These models have demonstrated particular utility in the identification of cell associated colonization factors, toxins, metabolic features, and regulatory pathways that contribute to disease (see Table 2.1). The immune pathways are well known within fruit flies and virulence factors such as cholera toxin, regulatory components like *CepR*, *pilF*, and *wspF*, and metabolic process like the purine biosynthetic pathway contributes to pathogen virulence (Kim et al., 2008; Schwager et al., 2013). The nematodes are high-throughput and have been utilized for the identification of virulence factors such as *dsbA*, *gacA*, and *rpoN*; as well as toxins such as *toxA*, alpha-hemolysin, serine protease, and cholera toxin (Cinar et al., 2010; Sifri et al., 2003; Tan et al., 1999). *M. sexta* was a successful infection model used in deciphering the pathogenesis of many pathogens such as *Serratia marcescens*, *Yersinia* sp.. (Fuchs et al., 2008; Petersen et al., 2012). This model has identified virulence factors such as the regulatory component *CpxRA* as well as toxins like metalloprotease (*Prt*) and toxin complement systems. The beneficial use of

this model is its ability to survive exposure to the human body temperature during infection assays.

An important consideration for the development of an alternative model to elucidate virulence by *V. parahaemolyticus* is to provide conditions, such as temperature or media, which are appropriate for expression of key virulence traits that contribute to human disease. Although few factors have been definitively linked to disease, those traits most associated with pathogenic potential include the hemolysin genes *tdh* and *trh*, and TTSS loci (and particularly TTSS2). Notably, hemolysin expression and production is strongly induced at human body temperature, 37°C (Mahoney et al., 2010). Finally, a comparative survey of *V. parahaemolyticus* strains implies that coordinated expression of virulence-associated traits in clinical strains may contribute to virulence. For this reason, a model that allows the investigation of interaction at higher temperatures may be particularly useful.

In order to gain insight of the virulence mechanism of *V. parahaemolyticus*, an infection model capable of emulating the infectious process that is readily available is necessary. We evaluated the capability of multiple invertebrate models, including *D. melanogaster*, *M. sexta*, and *C. elegans*, 1) to emulate *V. parahaemolyticus* infection and disease and 2) to reveal variation in pathogenicity among isolates of *V. parahaemolyticus* with expected differences in virulence. Among these invertebrate models, *C. elegans* demonstrated the most consistent, quantitative, and reproducible disease response. In addition, direct colonization competition assays can be conducted to determine whether pathogens are more virulent due to enhanced colonization. The development of a readily

available and inexpensive infection model will facilitate the identification of virulence factors within this emergent pathogen.

MATERIALS AND METHODS:

Bacterial strains and growth conditions. Strains used in this study with relevant characteristics are listed in Table 2.2. Hereafter, *B. cenocepacia* will be referred to as B.c., *P. aeruginosa* as P.a., *V. cholerae* as V.c., *E. coli* as E.c., and *V. parahaemolyticus* as V.p. The positive control strains B.c. HI2424 (Cooper et al., 2009), P.a. PA01 (Walker et al., 2004), and V.c. C6706 (Vaitkevicius et al., 2006) are pathogens that cause disease in alternative models such as *C. elegans*, and *D. melanogaster* (Blow et al., 2005; Cooper et al., 2009; Rahme et al., 1997). The non-pathogenic negative control E.c. OP50 is the normal food source for *C. elegans* (Brenner et al., 1973). In addition, six strains of *V. parahaemolyticus* were chosen to assess the ability of multiple models to distinguish virulence through infection (Table 2.2). *V. parahaemolyticus* and *V. cholerae* were grown in heart infusion (HI) broth at a pH of 7.3 (Fluka, Buchs, Switzerland) with shaking at 200rpm at both 28°C, a temperature used to cultivate environmental *Vibrio* spp. (31), and 37°C, the human body temperature. *E. coli*, *P. aeruginosa*, and *B. cenocepacia* were grown in Luria-Bertani (LB) broth (Sambrook et al., 1989) at 37°C for routine culturing, or at either 28 or 37°C for various assays. Antibiotics were supplemented into growth media as needed to maintain plasmids including Kanamycin (Km) at 50ug/ml for gfp labeling (pVSV102) and chloramphenicol (Ch) at 25ug/ml for rfp labeling (pVSV208) labeling.

Invertebrate hosts and growth conditions. Wingless *D. melanogaster* were obtained through Josh's Frogs, Owosso, MI and reared at room temperature on standard *Drosophila* media (Blow et al., 2005). For all infection experiments, flies were between 5 and 7 days old. *C. elegans* N2 was obtained from the *Caenorhabditis* Genetic Center, Minneapolis, MN. *C. elegans* N2 strain recovery was performed from a frozen state (Stiernagle et al., 2006) by scrapping ice crystals from the frozen stock and placing them onto square Petri plates (NC0262996, FisherSci) containing 50ml of nematode growth medium (NGM) consisting of 0.25% tryptone, 0.30% NaCl, 2% agar, potassium phosphate buffer at 1x (Stiernagle et al., 2006), 1mM CaCl₂, 1mM MgSO₄, and 0.1% of cholesterol (MP Biomedicals, LLC. Solon, OH). For culturing N2 strains, *E. coli* OP50 was seeded as a food source by spotting and spread-plating 500ul of liquid overnight culture grown at 37°C which was subsequently grown for 18 hours at 37°C prior to addition of N2 onto the NGM plate. Juvenile *M. sexta* and hornnematode dry food were obtained from Great Lakes Hornnematodes Washington, MI and maintained at room temperature until development to the 4th instar larvae stage. Hornnematode food was prepared by boiling 8 cups of water and mixing 1 lb. of hornnematode dry food. After solidifying, food was added to hornnematode habitat.

Fruit fly weight-loss measurements. Bacterial virulence within fruit flies was assessed based on the procedure as previously described (Blow et al., 2005) with additional modifications. Bacteria strains (Table 2.2) were grown as previously described. The OD₆₀₀ of overnight cultures were measured and the bacterial inoculums were standardized to 10⁸ cells per ml. A sterile cotton ball was placed at the bottom of each fly vial and inoculated with 1 ml of bacterial culture, or an LB control, each

prepared with 2.3 g/l tegosept to control growth of fungal contaminants. Fungal contaminants were found to occur with the use of LB and result in fly death. Week old adult flies were anesthetized using Flynap (Carolina Biological Supply Comp., Burlington, NC) and thirty flies per replicate were weighed using a PB403-S analytical scale (Mettler Toledo, Switzerland) and transferred to fly vials sealed with sterile foam corks, and maintained at room temperature (~25°C). After 24 hours, dead flies were removed by aspiration, and live flies were anesthetized and again weighed. All experiments were performed in triplicate and data reported as the average percent weight gain for live flies only. Significant differences between treatments were analyzed by a one-way ANOVA using the JMP Pro10 software package (SAS Institute Inc., Cary, NC).

Tobacco hornnematode infection assay. Infection of hornnematodes can be performed through direct injection methods, useful for addressing the virulence of whole-cell inocula, or oral toxicity assays, which can address the virulence of secreted toxins introduced into the digestive system of larvae (Hussa et al., 2012). Oral toxicity assays were chosen as the infection method in order to preserve the natural gastrointestinal route of infection by *V. parahaemolyticus*. Oral assays were performed based on a published protocol (Waterfield et al., 2001) with alterations to accommodate strain differences in this experiment. Select strains including, *P. aeruginosa* PA01, *V. cholerae* C6706, *V. parahaemolyticus* MDOH-04-5M732, and *E. coli* OP50, were used for initial assessment of hornnematodes as an infection model for *V. parahaemolyticus*. The OD₆₀₀ of overnight cultures were measured and standardized to an OD₆₀₀ of 0.3. Ten grams of solidified hornnematode food was saturated with one ml of culture, and allowed to dry for 10 minutes in experiment containers. The 4th-instar larvae were weighed and placed onto the

food block and incubated at 28°C and 37°C for 5 days. Hornnematodes were exposed to a 16 hour light and 8 hour dark cycle during experiments to promote regular development. Hornnematodes were limited to one per container to avoid cannibalism of dead hornnematodes. Mortality of hornnematode and weight of surviving larvae was recorded every 24 hours. Oral toxicity was assessed as the relative weight gain of infected animals compared to animals treated with a food control not treated with bacteria.

Synchronization of *C. elegans*. In order to synchronize the nematode populations to a common developmental stage, a standard bleaching method was applied, which kills adults and allows the recovery of eggs through centrifugation (Cooper et al., 2009). Nematode populations on 50 ml NGM agar plates were removed by the addition of 10.5 ml of ice-cold M9 buffer containing 3 g of KHPO_4 , 6 g of Na_2HPO_4 , 5 g NaCl, and 1 mM of MgSO_4 per liter. Nematodes suspended in M9 were collected into a conical tube with the addition of 3 ml of ice-cold bleach solution, containing 2 ml of household bleach and 1 ml of 5 M sodium hydroxide, was added to 9 ml of M9 with nematodes. Conical tubes were incubated in ice with 10 second vortexing at maximum speed every 2 minutes for 10 minutes. Eggs were then concentrated by centrifugation at 1,000 rpm for 7 minutes. Supernatant was removed by leaving ~1 ml to avoid disruption of the egg pellet. Egg pellet was resuspended with 2 ml of ice-cold M9 to wash residual bleach off the eggs and centrifuged at 1,000 rpm for 3 minutes. Two wash cycles were performed to minimize carryover of bleach. The eggs were plated onto a fresh 50 ml NGM plate seeded with *E. coli* OP50 and maintained at room temperature for approximately three days. Hatched nematodes were allowed to develop to the L4 stage as determined by the average growth cycle of nematodes (Baylis et al., 2012) before virulence assay performed.

Liquid substrate gastric virulence assays with *C. elegans*. Liquid killing assays for N2 nematodes were adapted from previous protocols (Cooper et al., 2009; Sellegounder et al., 2011) and performed as follows. Overnight bacterial cultures were diluted in M9 buffer to an OD₆₀₀ of 0.3 and one ml of culture or a buffer control placed in each well of a 48-well plate (NC0183674, FisherSci). Synchronized L4 nematodes were washed from the 50 ml NGM plate using M9 buffer. Nine ml nematode suspension was collected in conical tubes and isolated by centrifugation at 1,000 rpm for 7 minutes. Nematodes were washed with 2 ml M9 three times to limit *E. coli* OP50 cross-contamination. Countable numbers (~50) of synchronized L4 nematodes in 100µl volume were transferred into each well of the 24-well plate. The plates were incubated at ~25°C and observed at 4, 8, 24 hours for the rate of mortality. Nematodes were considered to be dead when they showed no response to external stimuli like a gentle tap or touch with a sterile needle.

Solid substrate gastric virulence assays with *C. elegans*. Virulence assays utilizing nematodes on solid agar were performed as previously described (Tan et al., 1999) with additional changes with each bacterial treatment in triplicate. See appendix for a detailed Standard Operating Protocol. Overnight cultures of each strain (Table 2) were grown at either 28°C or 37°C with shaking at 200 RPM. One-hundred µl of culture was spread as a 5 cm diameter circle in the center of each of three NGM plates, and grown for 24 h at the same temperature as overnight culturing. Stage L4 nematodes in 9 ml M9 buffer were collected and centrifuged at 1,000 rpm for 7 minutes. Nematodes were washed three times each with 2 ml of M9 to minimize bacterial carryover. Approximately 25 L4 nematodes suspended in 100 µl were transferred to each bacterial

lawn and incubated in their respective temperatures. Assay plates incubated at 37°C were transferred to 28°C after 6 hours to minimize adverse effects on nematodes. The rate of mortality was observed and recorded at 24 and 48 hours. Mobility rates of nematodes from various control and treatment conditions were also recorded on living nematodes by recording the time in seconds required for each of 15 nematodes per treatment to migrate 2 cm under a 1.5x magnification on the dissecting microscope. Significant differences in mobility rates were analyzed using a one-way ANOVA with the JMP software suite (version 10, SAS Institute Inc.). The utilization of PGS containing 10 g peptone, 10 g sodium chloride, 10 g glucose, 27.3 g d-sorbitol, and 17 g of agar per liter to promote virulence in pathogenic strains was also investigated as the solid substrate exactly as described for NGM. Notably, PGS is a richer medium with a higher osmolarity designed to enhance effects of secreted toxins (Tan et al., 1999).

Statistical Analysis. *C. elegans* mobility rates were plotted and the statistical significance of choice index between controls and *V. parahaemolyticus* strains was analyzed using one-way ANOVA and the all pairs Tukey-Kramer test in JMP Pro10 software package (SAS Institute Inc., Cary, NC).

Florescence labeling. *V. parahaemolyticus* was labeled for microscopy studies, by using plasmids expressing GFP (pVSV102) or RFP (pVSV208) introduced via conjugation as described (Stabb et al., 2002). In brief, recipient *V. parahaemolyticus* strains grown in HI medium at 28°C, and donor and helper *E. coli* (containing pEVS104) grown in LB medium at 37°C with the addition of appropriate antibiotics for selection for 18 hours were pelleted, washed, and combined in equal molar ratios in HI medium. Mating mixtures were spotted onto HI agar and incubated at 37°C for 4.5 hours. Mating

spots were then recovered, serially diluted with HI medium and plated onto HI agar medium containing appropriate antibiotics. Colonies formed after an overnight incubation at room temperature were observed visually for GFP or RFP production under an inverted fluorescent microscope. The bacteria were quadrant streaked for isolation, and grown in HI broth before being stored in 27% glycerol at -80°C..

Bacterial competition. Bacteria that were reciprocally tagged with Gfp or Rfp were competed in a pair-wise colonization assay. Strains were cultured in broth medium with appropriate antibiotic selection at 37°C. The OD₆₀₀ of overnight cultures were standardized to a starting OD₆₀₀ of 2.0. One ml of the each culture was transferred to a microcentrifuge tube, pelleted, and washed 3 times with M9 buffer. Competing strains were mixed, resuspended in 100 µl of HI media, and spread in a 5 cm diameter circle in the center of NGM plate. Synchronized nematodes (~50) were washed 3 times with M9 buffer and spotted 1 cm above the bacterial lawn. Plates were incubated at room temperature for 24 hours and colonization of nematodes viewed under the confocal microscope. Nematodes were also washed and homogenized to determine the colony forming unit of colonized bacteria.

C. elegans from the competition assays were washed 5 times with M9 buffer containing 1 mM sodium azide to inhibit bacterial expulsion from the nematode intestine and to remove bacteria superficially associated. One-hundred µl of solution containing approximately 30 nematodes were transferred to a microcentrifuge tube and placed in the -80°C for 15 minutes in order to kill the nematodes. Approximately 20 nematodes were placed onto a slide and the coverslip sealed with VaLaP sealant containing 13.8 g of vaseling, 13.8 g of lanolin, and 13.8 g of paraffin pellets. Bacterial localization within the

intestine of *C. elegans* exposed to fluorescently tagged bacteria was assessed using the confocal laser scanning microscope. The starting bacterial inoculum was calculated by washing the bacterial lawn off plates with 2 ml M9 buffer, serially diluting, and plating onto HI media containing the appropriate antibiotics and incubating at 28°C. The colony forming unit (CFU) of both the GFP and RFP labeled strains was calculated to determine the bacterial concentration the nematodes were exposed to.

Bacterial Colonization. The bacterial colonization of nematodes was quantified using a standard protocol (Sellegounder et al., 2011). Infected nematodes were collected from an NGM plate in 2 ml of M9 buffer containing 1 mM sodium azide. Nematode suspension was centrifuged at 1,000 rpm for 1 minute washed 5 times with M9 buffer containing 1 mM sodium azide. Slurry of approximately 20 nematodes was transferred to a microcentrifuge tube containing 400 mg of 1.0 mm silicon carbide particles (Biospec, Bartlesville, OK). Total volume was brought up to 250 µl with M9 buffer containing 1 mM sodium azide. Tubes were vortexed at maximum speed for 1 minute to homogenize the nematodes without affecting bacterial survival. The resulting homogenate was diluted and plated onto HI media containing Km to determine the CFU of the GFP and HI media with ch to determine CFU of the RFP strain.

RESULTS:

The *D. melanogaster* and *M. sexta* models of gastric infection by *V. parahaemolyticus* were unable to discriminate between virulent and non-virulent bacteria. The evaluation of the fruit fly *D. melanogaster* as a potential infection model

for *V. parahaemolyticus* was guided by the success of a published study involving *V. cholerae* (Blow et al., 2005). In that study, *D. melanogaster* lost weight after exposure to *V. cholerae* whereas it gained weight when exposed to LB broth containing no bacteria as the control treatment. The weight loss, presumed from diarrheal dehydration, required ingestion of cholera toxin; however, cholera toxin is only lethal when pathogenic isolates of *V. cholerae* are also ingested.

For our evaluation of the fruit fly model, we included a non-pathogenic reference, *E. coli* OP50 as a control since flies are fed bacteria and the addition of a non-pathogenic bacterium represents a more comparable control than LB, as well as, *V. cholerae* C6706 as a positive control (Fig. 2.1). Flies exposed to *V. cholerae* C6706 gained on average 0.2 mg, which was significantly less weight gain compared those exposed to no bacterial treatment (0.3 mg). Unfortunately, flies exposed to the non-pathogen *E. coli* OP50, gained an average of 0.25 mg. There was no significant difference between *V. cholerae* and *E. coli* treatments calling into question the utility of the model for discrimination between pathogens and non-pathogens, or for our purposes, virulence subtleties between pathogens of the same species.

To further scrutinize the applicability of the model, we increased the number of flies per replicate treatments to improve statistical power, and exposed them to a group of 10 select bacterial strains with expected varying virulence (Fig. 2.2.A). These strains include negative control non-pathogen *E. coli*, and several human pathogens that have broad virulence in other host models, including *B. cenocepacia* HI2424, *P. aeruginosa* PA01, and *V. cholerae* C6707. We selected a variety of *V. parahaemolyticus* strains, including pandemic (MDOH-04-5M732), non-pandemic clinical strains (AQ4037 and

Bac-98-03255), a pre-pandemic reference strain (BB22), an mutant derivative of BB22 with attenuated virulence in the mouse gastric model (GacA⁻) (Mahoney, 2011), as well as a representative environmental isolate lacking all proposed virulence markers (G61) (see Table 2.2 and methods for details). Typically, flies lost weight when exposed to pathogenic control strains, although their response was highly variable within cohorts and between experiments. For example, in a representative experiment presented in fig 2.2.B, only flies exposed to *P. aeruginosa* PAO1 lost weight, and flies exposed to *V. cholerae* C6706 on average gained weight. Furthermore, there was no significant difference between any treatments. The percent weight gain of flies treated with *V. parahaemolyticus* strains did not correlate with the pathogenic differences of these strains. Thus, the fruit fly model was unable to discern differences in virulence.

The tobacco hornnematode *M. sexta* was utilized in elucidating temperature regulation of virulence factors in *S. marcescens* (Petersen et al., 2012). Initial investigation of the hornnematodes was done by treatment with a select few strains including positive controls *P. aeruginosa* PAO1 and *V. cholerae* C6706, the pandemic *V. parahaemolyticus* strain MDOH-04-5M732, a negative bacterial control *E. coli* OP50, and a negative control of no bacteria. Hornnematodes infected through the gastrointestinal route were weighed every day up to day 7 and maintained at both 28°C and 37°C. Surprisingly, hornnematodes fed positive controls did not die from infection after 7 days at either temperature. Hornnematodes fed the *E. coli* OP50 and the food without bacteria also survived. In some instances, hornnematodes fed pathogen controls gained more weight than those fed non-pathogenic *E. coli* OP50 or untreated food. When hornnematodes were fed the *V. parahaemolyticus* strain, two out of six hornnematodes

developed diarrhea. However, within replicates, nematodes did not consistently lose or gain weight over the course of the experiment. The percent weight gain of hornnematodes treated with control strains did not correlate with the pathogenic differences of these strains. Thus, the hornnematode infection model was unable to discern difference between pathogenic and non-pathogenic strains.

The nematode *C. elegans* gastric infection model reveals variation in virulence of bacterial strains. The utilization of nematodes as a possible alternative model for *V. parahaemolyticus* infection studies was prompted from published studies in other bacterial species demonstrating its broad utility (Aballay et al., 2001; Dhakal et al., 2006) and a single study with *V. parahaemolyticus* (Sellegounder et al., 2011). In that study, nematodes were killed when fed with *V. parahaemolyticus* and nematode death was attributed to intestinal colonization and pharyngeal damage. In that study, as with ours, the negative control was *E. coli* OP50. However, that study did not utilize a defined pathogen positive control. Furthermore, the two strains of *V. parahaemolyticus* utilized, including strain ATCC17802 and a coral mucous associated isolate CM2 have undefined virulence in any model. One potential shortcoming of this model is that prolonged exposure to human body temperature is deleterious on their development; therefore, assays are typically conducted at 28°C or lower.

For our evaluation of the nematode model, we utilized the same 10 bacterial strains first described in fruit fly model (See methods for details). We anticipate at a minimum that the control pathogen treatments will cause death (Aballay et al., 2002; Sellegounder et al., 2011) and that the most virulent human pathogenic strains of *V. parahaemolyticus* (e.g. pandemic strains) would also kill nematodes. All nematodes fed

the pathogen controls *B. cenocepacia* HI2424 and *P. aeruginosa* PA01 died within 48 hours while those exposed to *V. cholerae* C6706 did not. Nematodes fed the non-pathogenic *E. coli* OP50 survived. Unexpectedly, nematodes fed clinical *V. parahaemolyticus* strains did not die.

After closer examination of nematodes, we observed differences in nematode mobility after treatment with various strains that was consistent with virulence. Specifically, after 24 hours, living nematodes fed pathogenic strains appeared less mobile than the nematodes fed non-pathogenic bacterial strains, which was quantifiable as mobility rate (Fig 2.3). The mobility rate of nematodes exposed to the positive control pathogens *B. cenocepacia* HI2424, *P. aeruginosa* PA01, and *V. cholerae* C6706 was significantly slower than the mobility of nematodes exposed to non-pathogenic (food) *E. coli* OP50. Nematodes exposed to various *V. parahaemolyticus* strains had motility that was intermediate to the pathogenic and non-pathogenic control treatments. As expected, the mobility of nematodes treated with the attenuated derivative of strain BB22 (GacA⁻) and presumed non-pathogenic environmental isolate of *V. parahaemolyticus* G61, was significantly faster than nematodes fed strains with known virulence properties. Among the pathogenic strains, the pandemic isolate MDOH-04-5M732 was the most virulent. Thus, the nematodes displayed a decreased mobility consistent with disease and variation in the severity of mobility impairment was consistent with expected differences in virulence properties among *V. parahaemolyticus* strains.

Temperature regulation plays a key role in *V. parahaemolyticus* virulence of the nematode *C. elegans* as a gastric infection model. Temperature effects the virulence expression of many bacteria species including *V. parahaemolyticus* (Mahoney

et al., 2010). An increase in temperature from 28°C to 37°C correlated with increased virulence during infection by *S. marcescens*, and enhanced the expression of several virulence-associated factors, such as hemolysin, protease, biofilm, and motility in clinical strains of *V. parahaemolyticus*; however, this trend was not conserved in environmental *V. parahaemolyticus* strains. Thus, temperature regulation may be a novel method to differentiate pathogenic strains from the harmless environmental strains.

In order to assess the effect of temperature on *V. parahaemolyticus* virulence, the nematode gastric infection model was performed at a constant temperature of 28°C, or at 37°C for 6 hours of treatment followed by maintenance at 28°C, for the purpose of evaluating temperature regulation of virulence. We utilized the same 10 bacterial strains first described in fruit fly model. At 37°C, nematodes fed the positive controls *B. cenocepacia* HI2424 and *V. cholerae* C6707 all died, while nematodes fed *P. aeruginosa* PA01 had significantly reduced mobility rate (Fig 2.3). Exposure to 37°C did decrease the mobility rate of nematodes fed the *E. coli* OP50 but mobility rate of nematodes was still significantly faster than the nematodes fed the pathogenic controls. Nematodes fed *V. cholerae* C6706 at 37°C all died, whereas they survived treatment at 28°C; however, the mobility rate was faster than the nematodes fed either *B. cenocepacia* HI2424 or *P. aeruginosa* PA01. At 28°C, the mobility rate of nematodes fed the clinical *V. parahaemolyticus* strains were significantly faster than at 37°C. However, the increased temperature did not cause a similar change in the mobility rate of nematodes fed the attenuated and non-pathogenic *V. parahaemolyticus* strains. Thus, human body temperature enhances virulence by clinical *V. parahaemolyticus* strains.

The PGS media did enhance bacterial virulence within the *C. elegans* gastric infection model but did not enhance death of the nematodes. In previously published studies with *B. cenocepacia* and *P. aeruginosa*, the use of PGS media increased pathogen virulence and caused death in nematodes (Cooper et al., 2009; Hendrickson et al., 2001). PGS is a richer, high osmolarity medium designed to enhance effects of secreted toxins. In the study utilizing *P. aeruginosa* study, this media resulted in nematode death from bacterial infection within 12 hours, whereas NGM media resulted in nematode death between two to three days.

In an effort to develop an infection model with definitive results such as death from feeding with pathogens that would be less laborious than measuring mobility, the nematode gastric infection model was evaluated on PGS medium. The use of PGS in the nematode infection assay was compared to NGM at 24 hours at 28°C (Fig 2.4). On PGS the nematodes fed the positive controls *B.cenocepacia* HI2424, *P.aeruginosa* PA01, and *V. cholerae* C6706 all died, while the nematodes fed the negative control *E. coli* OP50 had a reduced mobility rate. Unexpectedly, the use of this assay media did not result in death of nematodes fed the pathogenic *V. parahaemolyticus* strains. The nematodes fed the pathogenic strains *V. parahaemolyticus* MDOH-04-5M732, *V. parahaemolyticus* BB22, and *V. parahaemolyticus* BAC-98-03255 had significantly reduced mobility rates. The nematodes fed the attenuated derivative of strain BB22 (GacA⁻) had a reduced mobility rate that did not differ from those exposed to pathogenic strains, suggesting this medium may interfere with discrimination of subtle differences in virulence that may be observed with mutant bacteria. Nematodes fed the presumed non-pathogenic environmental strain G61 had no change to their mobility rate. The use of PGS did

enhance the virulence of pathogenic strains as well as the attenuated and negative control strains; however, this media did not enhance strain virulence enough to cause death and therefore does not facilitate a more high-throughput assay.

The colonization of the nematode *C. elegans* intestine during infection varies among *V. parahaemolyticus* strains. In a previous study using liquid colonization assays, nematodes colonized with strains of *V. parahaemolyticus* of undefined virulence results in gross distention in the post pharyngeal region with notable pharyngeal damage, while *E. coli* was unable to colonize the intestine and caused no morphological changes (Sellegounder et al., 2011).

In order to evaluate whether the mobility rate changes in *C. elegans* fed various bacterial treatments correlated with pathogen colonization, nematodes fed fluorescently-labeled bacteria were examined by scanning laser confocal microscopy. A CLSM image of *C. elegans* with arrows indicating anatomical locations is shown in figure 2.5. The location in the gastrointestinal tract and the density of bacterial cells *V. parahaemolyticus* strains BB22, GacA⁻, MDOH-04-5M732, and G61, varied within *C. elegans* (Fig 2.6). *V. parahaemolyticus* BB22 was mainly localized to the median bulb and cells decreased as the length of the intestine increased from the mouth (Fig 2.6A and 2.6B). Colonization by the attenuated strain of BB22 (GacA⁻) occurred mainly near the pharynx with only a few bacteria sporadically visible throughout the length of the intestinal tract (Fig 2.6C and 2.6D). MDOH-04-5M732 was visible throughout the entire length of the gastrointestinal tract (Fig 2.6E and 2.6F). G61 was isolated to areas near the median bulb and rarely within the intestinal tract (Fig 2.6G and 2.6H). The colonization of nematodes exposed to select *V. parahaemolyticus* strains were plated to obtain the CFU bacterial count within

nematode intestine. Nematodes exposed to GacA⁻ and MDOH-04-5M732 were colonized with an average CFU of 10³ per nematode after 24 hours, whereas the average CFU of BB22 and G61 was about 10² per nematode.

MDOH-04-5M732 has a greater fitness in colonization during pair-wise competition with non-pandemic strains in nematodes. In order to better determine differences between colonization, direct pair-wise competitive colonization was conducted between the pandemic strain MDOH-04-5M732 and a pre-pandemic strain BB22. Competitive index, where the final bacterial concentration and ratio of strains is normalized by the starting ratio is used as an additional sensitive measure for colonization ability. In the pair-wise competition of GFP-tagged BB22 and RFP-tagged MDOH-04-5M732 (Table 3, Fig 2.7A and 2.7B), even though nematodes were exposed to 40 times more BB22 than MDOH-O4 cells within the inoculum, MDOH-04 exhibited remarkable dominance with a competitive index of 900, indicative of its enhanced colonization ability.

DISCUSSION:

In order to properly function as infection models, the model system must mimic both the infection process and the host response. Numerous studies of *V. parahaemolyticus* virulence mechanisms have been assessed in models such as mice, piglets, and rabbit ileal loops (Boutin et al., 1979; Caburlotto et al., 2010; Hiyoshi et al., 2010; Pineryo et al., 2010). Even when the use of these models is justifiable, these models cannot be used in large scale high-throughput infections to assess virulence

potential of *V. parahaemolyticus* strains. Our previous studies utilizing infant mice also showed high levels of variability between replicate experiments (Mahoney 2011). The lack of a fully developed immune system allowed bacterial colonization but does not represent the true interaction between a pathogen and the host immune system. In addition, the developmental stages of mice was crucial since more developed mice become symptomatic but are able to fully recover (Mahoney et al., 2011). Cytotoxicity against human cell lines was also explored as an alternative to animal models, but a recent study indicates that presumably non-pathogenic strains lacking all known virulence factors are still highly cytotoxic in these assays. For these reasons, analysis of other potential host models is warranted.

In this study, we assessed multiple alternative models in *V. parahaemolyticus* infections and their ability to distinguish different virulence potentials of select strains, and most of these models failed to discriminate between strains with varying pathogenicity (Table 1; Table 2). Although *D. melanogaster* shows promise for use with *V. cholerae* (Blow et al., 2005), our results indicate an unacceptable level of variability between replicate treatments and an inability to discern pathogens from non-pathogenic bacteria by this model (Fig 2.1, 2.2). This difference may be attributed to the difference in controls used in our study; specifically the inclusion of a non-pathogenic bacterial control, *E. coli* OP50, rather than only an LB broth control. The ability of the tobacco hornnematode to survive at both 28°C, the normal growth temperature of *V. parahaemolyticus*, and 37°C, the human body temperature, would allow for the elucidation of virulence regulated by temperature (Mahoney et al., 2010). However, when

bacteria were introduced with food, weight gain between nematodes and accompanying symptoms of diarrhea were highly variable.

In a single previous study, strains of *V. parahaemolyticus* with undefined virulence properties infected and killed *C. elegans* in a liquid infection assay (Sellegounder et al., 2011). However, in studies presented herein, *C. elegans* death did not occur after exposure to known human pathogenic strains of *V. parahaemolyticus* in either a solid assay (Fig. 2.3) or a liquid assay (data not shown). The lack of death is most likely due to the difference in *V. parahaemolyticus* strains used in this study. However, virulence differences were observed within infected nematodes when assessed by mobility rates (Fig 2.3). Nematodes fed on pathogenic *V. parahaemolyticus* strains showed a significantly slower mobility than nematodes fed on *E. coli* OP50. More importantly, variation in mobility correlated with expected differences in virulence (Fig 2.2A). Although attempts to optimize the assay by enhancing sensitivity of the nematodes was attempted by the use of PGS media, these did not lead to death of nematodes treated with pathogenic strains of *V. parahaemolyticus* and obscured subtle differences between strains as seen by comparisons between BB22 and its attenuated derivative GacA⁻ (see Fig 2.4).. The mobility rate of nematodes can be a useful tool in measuring virulence of strains not able to cause death from infection. *V. parahaemolyticus* is able to colonize nematode intestine and this accumulation of bacteria leads to distension of the gastrointestinal tract shown in a previous publication (Sellegounder et al., 2011). In that study, *V. parahaemolyticus* infection induced a strong inflammatory response in the host intestine determined by real-time PCR may lead to the reduced mobility rates. Another pathogen of the *Vibrionaceae* family, *V. cholerae* C6706 also reduced the mobility rate of

infected nematodes. In order to gain a better understanding of this novel host response for determining virulence, further studies should be done with the pathogen *V. cholerae* where the virulence mechanisms are better understood.

Previous studies imply temperature regulation may play a role in the virulence of *V. parahaemolyticus* which holds promise for aiding in the understanding of disease mechanisms through their differential regulation (Mahoney et al., 2010). Specifically, in a comparison of clinical and environmental isolates, there was a significant correlation of the production of virulence-associated factors with human body temperature (Mahoney et al., 2010). This temperature regulation of virulence was further characterized here within the nematode infection model. Significant increase in virulence based on mobility was observed in pathogenic strains when exposed to the human body temperature based on nematode mobility, whereas a non-pathogenic *V. parahaemolyticus* strain did not have altered virulence at the higher temperature of 37°C compared to 28°C consistent with the hypothesis that coordinated induction of acquired and core virulence factors contributes to disease. However, it is worth noting that G61 is an environmental isolate from NH, and as a potentially cold water adapted isolate may have unique temperature regulation compared to similar environmental isolates from warmer regions. Thus, virulence regulated by temperature needs further investigation from among a more diverse sample of environmental isolates to determine if the lack of temperature response is unique to strains from colder climates with the addition of environmental strain from warmer climates.

In an attempt to develop a more high-throughput nematode model, conditions that promote strain virulence were also tested by changing media. Previous studies showed

that the use of PGS agar, a richer and higher osmolarity medium, enhanced effects of secreted toxins (Caburlotto et al., 2010; Rahme et al., 1999). An increase in virulence was observed within infected nematodes when the assay was performed on this media; however, the use of this media was not ideal for the purpose of this study. With the use of this media, the virulence of non-pathogenic strains increased as well as the pathogenic strains. This may mean that non-pathogenic strains can produce small amounts of toxin, which when enhanced on this media causes a detrimental effect on the nematodes. In addition, death from pathogenic *V. parahaemolyticus* strains was not seen and the separation between the more virulent pandemic strain and the other pathogenic strains was no longer observed (Fig 2.4). On the basis of these results, the use of this media condition did not achieve the goal of a high-throughput nematode virulence assay.

The colonization of *V. parahaemolyticus* in *C. elegans* intestine was localized using the CLSM to determine if reduced mobility correlated with intestinal colonization. Nematodes infected with *V. parahaemolyticus* showed an extensive colonization of bacteria along the intestinal tract with the exception of the NH environmental isolate (Fig 2.6). Like previous reports (Sellegounder et al., 2011), this colonization was not observed in nematodes fed with *E. coli* OP50 (data not shown). The intensity of colonization was measured through the plating of infected nematodes by the bacterial colonization assay. After 24 hours of infections, the CFU/ml was between 10^3 and 10^4 depending on the strain. The strains with lower bacterial counts correlated to reduced fluorescence seen within infected nematodes by SLCM. Direct competitions between a pandemic and a pre-pandemic strains further emphasized the enhanced colonization ability of the pandemic

strain. The ability of the pandemic strain to dominate at a ratio of 900:1 in competition and the lack of visible GFP labeled BB22 in *C. elegans* intestine is supported by previous studies showing that these strains are more virulent and are able to become the dominate serotype in a given area (Nair et al., 2007).

The nematode as a gastric infection model for *V. parahaemolyticus* has shown great promises in being able to emulate disease and elucidate the virulence mechanism of this emergent pathogen. This model is currently not able to screen through thousands of mutants at a high-throughput rate since mobility rates takes time to assess from nematode to nematode. Thus, the development of a response such as death from infection would improve the use of this model. This model is also only able to survive high temperatures such as 37°C for about 8 hours. However, this is still useful for assessing the temperature regulation of *V. parahaemolyticus*. Even though some effects may be due to nematode stress from the temperature exposure, it was shown by the nematodes fed the *E. coli* OP50 that they are not largely affected by this temperature for short periods of time. This model is capable of showing subtle differences in virulence which would be necessary in a mutagenic screen and can also show whether a particular environmental isolate is more similar to a pathogen.

Table 2.1. Alternative models and the virulence mechanisms identified.

Strain	Identified virulence factor	Reference
<i>A. thaliana</i>		
Metabolism		
<i>P. aeruginosa</i>	<i>dsbA</i>	Rahme et al.
Regulatory		
<i>E. faecalis</i>	<i>fsrB</i>	Jha et al.
<i>P. aeruginosa</i>	<i>gacA</i>	Rahme et al.
	<i>rpoN</i>	Hendrickson et al.
Toxin		
<i>E. faecalis</i>	<i>serine protease sprE</i>	Jha et al.
<i>C. elegans</i>		
Metabolism		
<i>P. aeruginosa</i>	<i>dsbA</i>	Tan et al.
Regulatory		
<i>P. aeruginosa</i>	<i>gacA</i>	Tan et al.
	<i>rpoN</i>	Hendrickson et al.
<i>S. aureus</i>	<i>Agr</i>	Sifri et al.
	<i>sarA</i>	Sifri et al.
Toxin		
<i>P. aeruginosa</i>	<i>toxA</i>	Tan et al.
<i>S. aureus</i>	<i>alpha-hemolysin</i>	Sifri et al.
	<i>V8 serine protease</i>	Sifri et al.
<i>V. cholerae</i>	<i>cholera toxin</i>	Cinar et al.
	<i>hlyA</i>	Cinar et al.
<i>D.melanogaster</i>		
Metabolism		
<i>B. cenocepacia</i>	Purine biosynthetic pathway (<i>purAFLD</i>)	Schwager et al.
Regulatory		
<i>B. cenocepacia</i>	<i>CepR</i>	Schwager et al.
<i>P. aeruginosa</i>	<i>pilF</i>	Kim et al.
	<i>wspF</i>	Kim et al.
Toxin		
<i>V. cholerae</i>	<i>cholera toxin</i>	Blow et al.
<i>M. sexta</i>		
Regulatory		
<i>Xenorhabdus</i> <i>nematophilia</i>	<i>CpxRA</i>	Tran et al.
Toxin		
<i>Photorhabdus</i> <i>luminescens</i>	<i>Prt</i>	Daborn et al.
	<i>Tcs</i>	Daborn et al.
<i>Xenorhabdus</i> <i>nematophilia</i>	<i>XhIA</i>	Cowles et al.

Table 2.2. Bacterial strains with relevant characteristics and expect effects used in this study.

Strain	Origin	Characteristic	Previous Models	Expected Outcome	Source
(+) Controls					
<i>B. cenocepacia</i> HI2424	Soil (USA, NY)	Insect and human pathogen	<i>C. elegans</i>	Disease/death	Cooper et al
<i>P. aeruginosa</i> PA01	Wound (Australia)	Insect and human pathogen	<i>C. elegans</i> , <i>Arabidopsis</i> , <i>D. melanogaster</i> , mice	Disease/death	Walker et al
<i>V. cholerae</i> C6706		Human pathogen	<i>D. melanogaster</i> , mice	Disease/death	Vaitkevicius et al
(-) Control					
<i>E. coli</i> OP50		Food/ non-pathogenic	<i>C. elegans</i>	No Disease	Brenner et al
<i>V. parahaemolyticus</i> treatments					
MDOH-04-5M732	Patient (USA, FL)	Highly virulent clinical pandemic strain	Mice	Disease	Davis et al
BAC-98-03255	Patient (USA, NY)	Virulent clinical strain		Disease	Myers et al
AQ4037	Patient (Japan)	Virulent clinical strain		Disease	Davis et al
BB22	Water (India)	Virulent pre-pandemic strain	Mice	Disease	McCarter et al
GacA ⁻ mutant of BB22		Attenuated clinical strain	Mice	Attenuated Disease	Mahoney 2010
G61	Water (USA, NH)	Typical environmental strain from NH		No Disease	Striplin et al

Table 2.3. *In vivo* competition assays of *V. parahaemolyticus*

Strain	Starting	Ratio	Ending	Ratio	CI ^a
MDOH-04-5M732	3×10^8		9.9×10^4		9×10^2
X		2.4×10^2		2.2×10^1	
BB22	1.23×10^{10}		4.5×10^3		

^aStrains were labeled in reciprocal fluorescence and no differences were observed

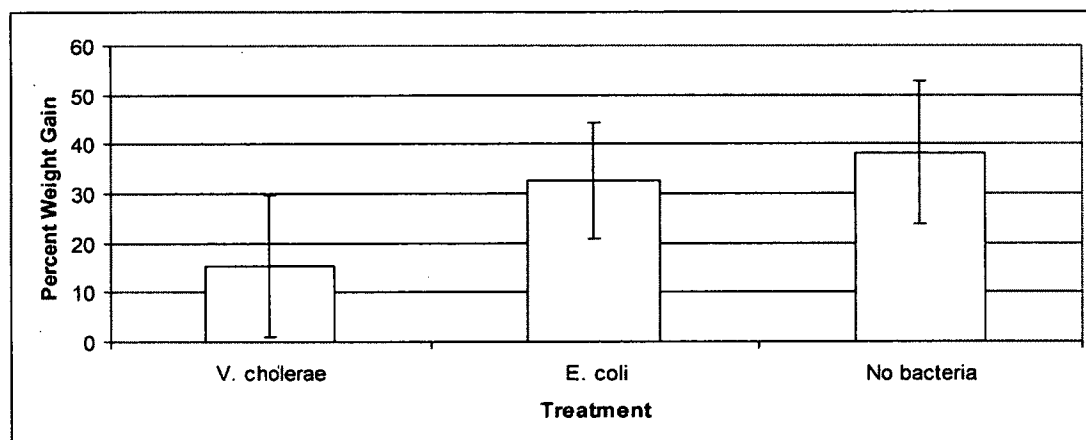


Figure 2.1: Percent weight gain of *D. melanogaster* after bacterial exposure and LB control. Thirty week-old adult flies were used and perform in triplicates. Error bars show the 95% confidence interval. No significance between treatments based on one-way ANOVA (All pairs).

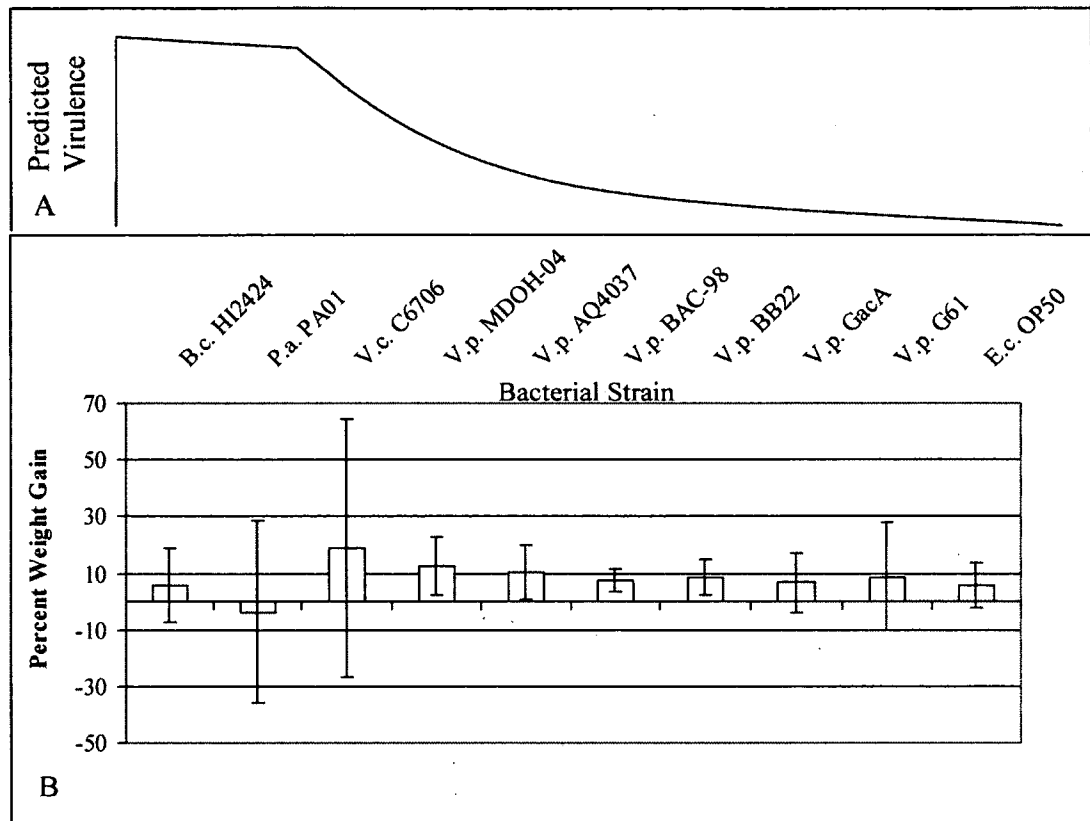


Figure 2.2. Percent weight gain of *D. melanogaster* post bacterial exposure to 10 select strains. (A) The expected virulence distribution of the 10 bacterial strains in animal model. (B) The actual virulence in *D. melanogaster* exposed to the 10 select bacterial treatments. Error bars show the 95% confidence interval of the triplicate experiments. No significance between treatments based on one-way ANOVA (All pairs).

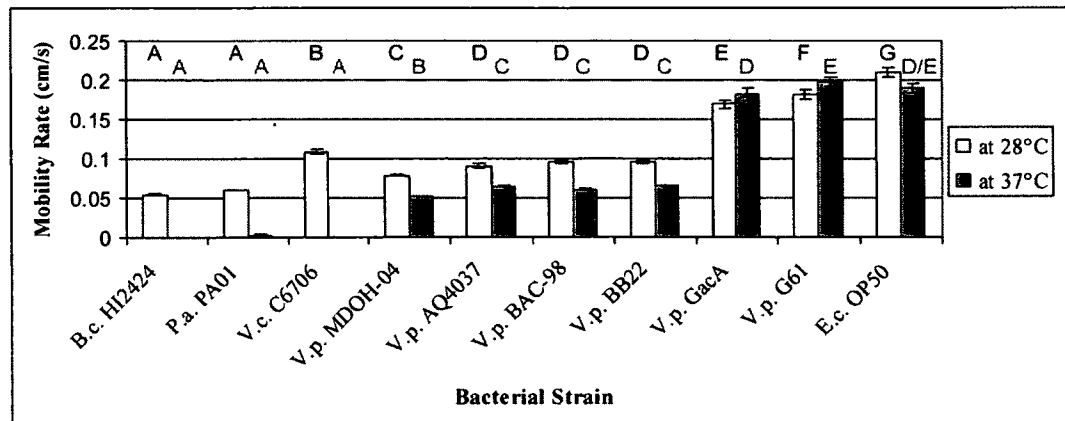


Figure 2.3. *C. elegans* mobility exposed to bacterial strains at 28°C and 37°C measured after 24 hours. *C. elegans* were exposed to different bacterial lawns on nematode growth medium (NGM). *C. elegans* were scored on the amount of time in seconds it takes to migrate 2 cm under a dissecting scope set to an objective of 1.5x. Mobility rate of *C. elegans* was calculated and no bars indicate death of the nematodes. Assays were performed in triplicates and error bars show the 95% confidence interval between nematodes scored. Letters show the significance at 28°C (black) and 37°C (gray) determined by one-way ANOVA (All pairs).

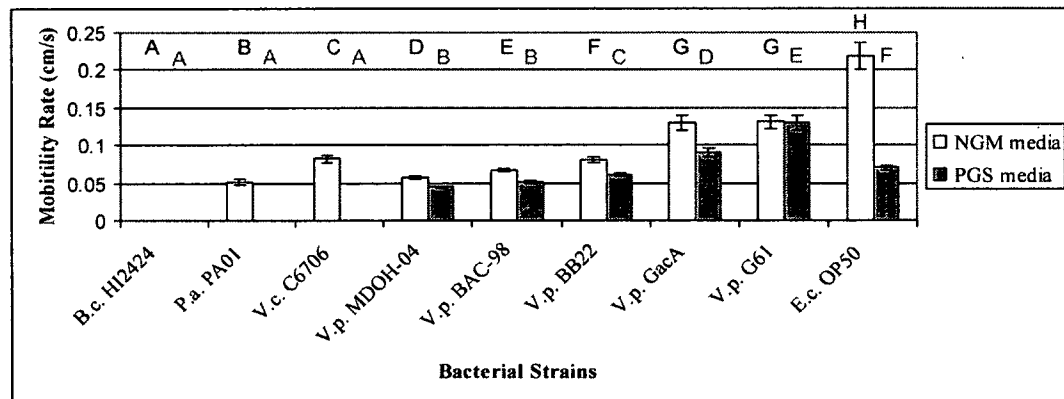


Figure 2.4. *C. elegans* mobility at 28°C after 24 hr exposure to bacterial strains plated on NGM and PGS. NGM is the normal growth media for nematodes and PGS promotes bacterial virulence by concentrating diffusible toxins. *C. elegans* mobility rate was calculated and the lack of bars indicates death of nematodes and mobility scoring was not possible. Error bars show 95% confidence interval between nematodes scored. Letters show the significance on NGM (black) and PGS (gray) determined by one-way ANOVA (All pairs).

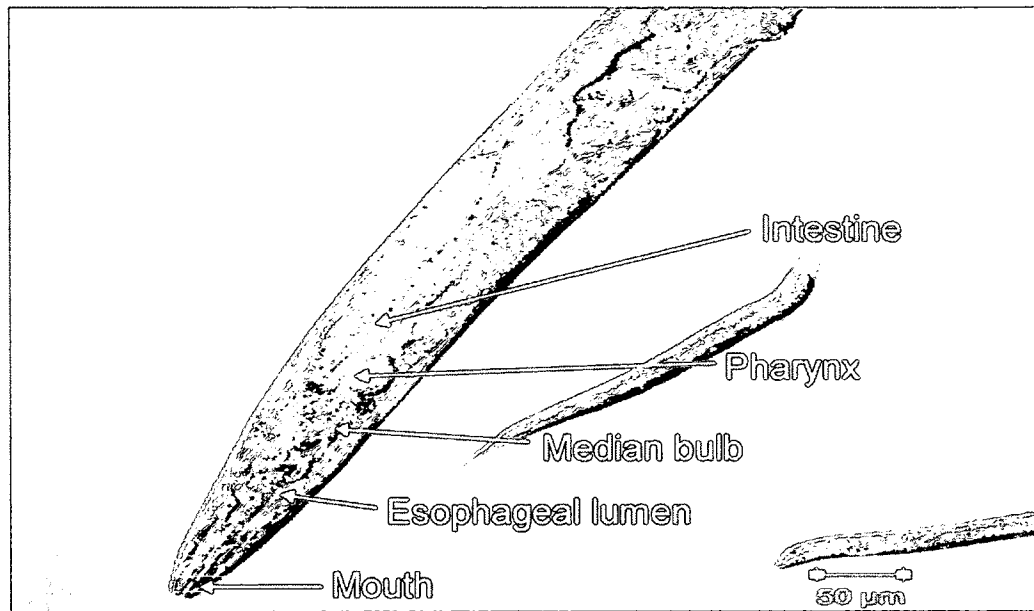


Figure 2.5. CLSM image of *C. elegans* with arrows indicating anatomical locations.

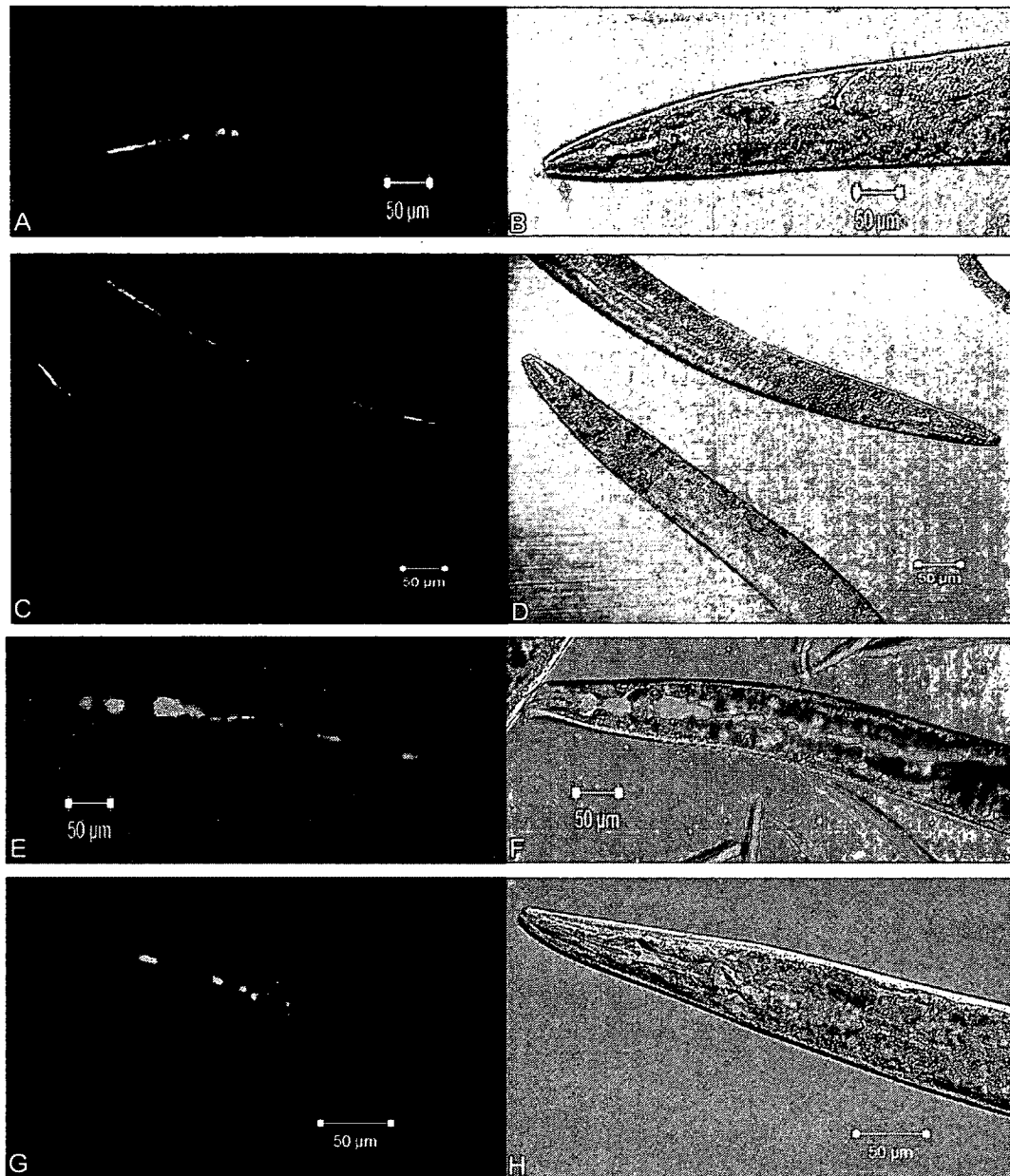


Figure 2.6. Colonization of GFP tagged *V. parahaemolyticus* within *C. elegans* gastrointestinal tract. CLSM images of *C. elegans* exposed to BB22 (A and B), attenuated BB22 mutant GacA (C and D), MDOH-04-5M732 (E and F), and G61 (G and H).

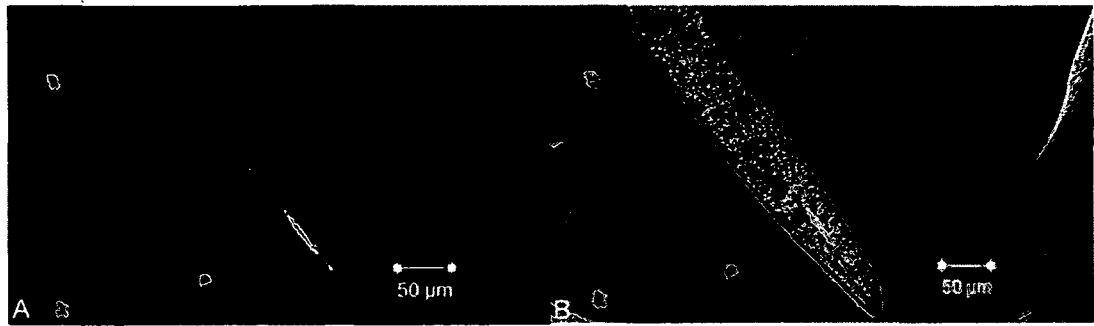


Figure 2.7. Pair-wise competition of *V. parahaemolyticus* pandemic and pre-pandemic strains in *C. elegans* intestine. CLSM image of colonization in *C. elegans* fed RFP tagged MDOH-04-5M732 and GFP tagged BB22 (A and B).

Chapter III

Identification of Putative Virulence-Associated Genes Through Transposon Mutagenesis

ABSTRACT:

V. parahaemolyticus is a human pathogen often associated with shellfish and whose virulence mechanisms are not well known. The presence of the hemolysin genes as markers to differentiate pathogens from harmless environmental strains is unreliable due to the presence of pathogenic strains lacking these genes. In addition, loss of these genes does not reduce virulence within mice. To elucidate traits associated with virulence, we generated 1,760 mutants through random transposon mutagenesis. These mutants were screened for phenotypes that contribute to virulence in other pathogens, such as biofilm, siderophore, hemolysin, motility, protease, and capsule. Hemolysin, motility, and protease have enhanced activity at 37°C compared to 28°C in *V. parahaemolyticus* so these traits were screened at both temperatures. We identified 576 mutants displaying altered phenotypes with 97 non-pleiotropic mutants. Twenty-two capsule mutants that were further analyzed in more detail in the *C. elegans* gastric model all exhibited reduced virulence in the *C. elegans* model, which correlated with reduced colonization ability, demonstrating that capsule plays a role in *V. parahaemolyticus* virulence. Continued research aimed at understanding the virulence mechanism of this emergent global pathogen will aid in future preventative measures.

INTRODUCTION:

V. parahaemolyticus is a marine bacterium that is the leading cause of bacterial food-borne gastroenteritis worldwide (Garcia et al., 2009). Infections of *V. parahaemolyticus* are often acquired through the consumption of raw or undercooked shellfish (Garcia et al., 2009; Park et al., 2004). Upon intestinal colonization clinical symptoms of infection occur including watery diarrhea, abdominal cramps, nausea, and vomiting (Daniels et al., 2000). Not all strains of *V. parahaemolyticus* are human pathogens and the high incidence of infections is attributed to the general lack of knowledge of *V. parahaemolyticus* pathogenesis that is necessary for identification of potential human pathogenic strains in shellfish (Broberg et al., 2011).

Only a few virulence genes are routinely used to identify pathogenic strains of *V. parahaemolyticus* although none are reliable for detection of all pathogenic strains. Among these, the most common include two hemolysin genes, thermostable direct hemolysin TDH toxin (*tdh*) gene, and the TDH-related hemolysin (*trh*) gene. Although the *tdh* and *trh* genes are widely accepted as virulence markers in strain typing, the identification of pathogenic strains by the presence of these genes is not always reliable (Broberg et al., 2011). An abundance of bacteria with these markers do not always correlate to outbreaks (Paranjpye et al., 2012). There have also been outbreaks caused by strains that do not contain either *tdh* or *trh* (DePaola et al., 2003). Mutant strains lacking hemolysin genes still causes fluid accumulation within the rabbit ileal loop model (Kodama et al., 2008) and are lethal in mice (Hiyoshi et al., 2010). At a minimum, these studies indicate these genes are not the only factors determining *V. parahaemolyticus* virulence.

A variety of factors or traits other than hemolysins and T3SS2 have been implicated as possible virulence determinants such as proteases, biofilm, siderophores, and capsular polysaccharide, based on precedents in other pathogenic bacteria including *Vibrio* spp. (Dhakal et al., 2006). Extracellular protease of *V. cholerae* facilitates the action of cholera toxin, whereas *V. vulnificus* produces metalloprotease which causes hemorrhagic reactions by degrading type IV collagen in basement membranes (Booth et al., 1984; Miyoshi et al., 1998). Siderophores have been indicated to play a role in virulence since the production of these iron chelators may provide strains with a competitive survival advantage in iron-limiting environments. When *V. parahaemolyticus* is grown under iron-limited conditions, there is an increase observed with hemolytic activities, adherence, and proliferation rates (Dai et al., 1992; Wong et al., 1994). In *V. vulnificus*, the capsule polysaccharide (CPS) expression is related to lethality seen within animal models (Chatzidaki-Livanis et al., 2006). Translucent colonies with decreased CPS expression have diminished virulence in mouse models (Wright et al., 1990). These virulence traits are established in other organisms but their role in disease of *V. parahaemolyticus* is uncertain. However, the expression of some of these traits is regulated by a temperature shift from 28°C to 37°C. Hemolysin, protease, and biofilm production in clinical isolates increases in response to the human body temperature (Mahoney et al., 2010).

In order to identify traits which contribute to the virulence of *V. parahaemolyticus*, we employed a genetic screen employing transposon mutagenesis (Hayes et al., 2003). A total of 1,760 random transposon mutants were generated and screened for phenotypic changes in traits that potentially contribute to virulence including

protease activity, hemolytic activity, biofilm formation, siderophore, and capsular polysaccharide. Twenty-two apparently non-pleiotropic mutants were identified with reduced capsule production. ,Because capsule is an important virulence factor in the related pathogen *V. vulnificus* (Wright et al., 1990) due to its contribution to survival and immune evasion, and capsule mutants in that organism are impaired in mice, these non-pleiotropic mutants were selected for further analyzed for their virulence and colonization in a recently optimized nematode model (Chapter 2). All twenty-two mutants had reduced virulence as assessed by nematode mobility. Reduced virulence correlated with reduced colonization ability as determined by direct competition of two of these capsule mutants against wild-type MDOH-04-5M732 utilizing fluorescence microscopy.

MATERIAL AND METHODS:

Bacterial strains and growth conditions. Bacterial strains and plasmids are listed in table 3.1. *V. parahaemolyticus* strain MDOH-04-5M732, a clinical pandemic isolate kindly provided by J. Cattani (Davis et al., 2007), was routinely cultured in heart infusion (HI) broth at a pH of 7.3 (Fluka, Buchs, Switzerland) with shaking at 200rpm at both 37°C and 28°C. *E. coli* DH5α were grown in Luria-Bertani (LB) broth (Sambrook et al., 1989) at 37°C. Antibiotics were supplemented into growth media as needed to maintain plasmids in donor (pEVS170) and helper (pEVS104) *E. coli* DH5α with the addition of Kanamycin (Km) at 50ug/ml for transposon mutagenesis. Generated mutants were stored in polypropylene microplates (Costar, St. Louis, MO) with 27% glycerol at -80°C after overnight growth in HI with shaking at 200 RPM at 28°C. For phenotypic

assays, mutants were grown in 96-well polystyrene microplates (Costar, St. Louis, MO) containing 200 μ l HI per well at both 28°C and 37°C.

Transposon mutagenesis. Transposon mutants were generated as previously described (Lyell et al., 2008). Briefly, the plasmid pEVS170 (donor) was transferred to MDOH-04-5M732 by triparental matings with the aid of the pEVS104 (helper) plasmid (Stabb et al., 2002). *V. parahaemolyticus* MDOH-04-5M732 was cultured in HI broth for 2 hours and then washed, mixed in equal molar ration with donors and helpers grown overnight, and spotted onto a HI plate for mating. Following a 4.5 hour incubation, mating spots were recovered, and resuspended in HI and dilution plated onto HI agar plates supplemented with 12.5 μ g/ml of erythromycin (Erm) selective for the transposon. Plates were incubated overnight at 28°C and colonies were isolation-streaked on HI with Erm. In order to ensure that erythromycin-resistant colonies carried the transposon insertion and were not harboring pEVS170, isolated mutants were also screened for kanamycin resistance by patching colonies on HI Kan 100, and kanamycin-resistant mutants were discarded. The Erm-resistant mutants were arrayed in 96-well polypropylene microplates (Costar, St. Louis, MO) with 27% glycerol for storage and then assays were performed by replicating with a 96-pin replicator (V&P Scientific Inc. San Diego, CA).

Characterization of virulence-associated phenotypes . Qualitative plate assays were performed for motility, capsule, and auxotrophy, and quantitative assays performed for protease, hemolysin and siderophore from seed cultures grown in HI with shaking for 18 hours prior assay. Motility was measured using procedures previously described (Jacques et al., 2006) with 50-ml soft agar plates containing 10 g tryptone and 20 g NaCl

in addition to 4 g Bacto agar per liter. Triplicate plates were inoculated with 10 µl of each culture using a 96-pin replicator (V&P Scientific Inc. San Diego, CA) in triplicate. After 18 hours, the diameter of the zone of mutant bacterial migration was visually compared to the wild-type migration on the same plate. Capsule variations between mutants were screened in triplicate on 50 ml congo red agar plates containing 10 g tryptone, 20 g NaCl, 0.2 g Congo red, and 7.5 g Bacto agar per liter spotted with 10 µl of each culture using a 96-pin replicator (V&P Scientific Inc. San Diego, CA). The plates were incubated at 28°C, and observed at 24, 48, and 72 hours and observed for darker red colonies compared to wild-type due to a reduced capsule production and cells being more translucent. Auxotrophs were identified using IM medium containing 500 ml 2x all purpose seawater, 50 ml Tris (pH 7.5), 15 g agar, 1 mM Phosphate stock, and 5 ml of 1 M ammonia stock per liter with either 0.2% glucose or 0.3% glycerol.

Quantitative siderophore assays were performed to determine the ability of generated mutants to produce the iron acquisition chelators. The chrome azurol S standard protocol was performed with slight modifications (Alexander et al., 1991). In brief, 1 µl of overnight cultures were inoculated into 200 µl of iron-free Pimm's minimal medium and incubated for 15 hours with shaking at 200 rpm. The OD₆₀₀ of the overnight PMM culture was determined using a Tecan infinite M200 plate reader (Mannedorf, Switzerland). Cultures were pelleted with centrifugation at 4,000 rpm (3,670Xg) for 15 minutes and 100 µl of supernatant was combined with 100 µl of CAS assay solution in a new polystyrene microplate. CAS solutions are made fresh in 50 ml total volumes for each assay. Solution A consist of 2.15 g piperazine with 15 ml milliQ water and 3.4 ml HCl. Solution B is made using 0.011 g Hexadecyltrimethylammonium (HDTMA) and 25

ml milliQ water. Lastly, Solution C is comprised of 0.75 ml Iron III solution and 3.75 ml 1x CAS solution. The order of mixing the 3 solutions is crucial with solution C added to B and then solution A is mixed in to solution B/C and the final addition of 1 ml shuttle solution which contains 0.0436 g 5'-sulfosalicylic acid. Assay plates are kept in the dark at room temperature for 1 to 1.5 hours and then read at an optical density of 630. It was seen that 1 hour was the minimum time required for the development of the assay.

Biofilm assays were performed according to a standard protocol (O'Toole et al., 1999), with slight modifications. Strains were inoculated into 150 μ l of HI medium per well of a polystyrene microplate (Costar, St. Louis, MO) and grown for 18 hours at 37°C with shaking at 200 rpm. Eighteen hour was empirically determined to be the point at which biofilm could be reliably measured without biofilm "clumps" disrupting the well and inducing significant variation. Once the OD₆₀₀ was determined using a Tecan infinite M200 plate reader (Mannedorf, Switzerland), the cultures were expelled from the plate, which was allowed to dry for 10 minutes, and incubated at 80°C for 30 minutes to affix the cells. After incubation, plates was washed twice with 300 μ l of distilled water per well. The plates were allowed to dry and 200 μ l of 0.1% crystal violet was added per well for 20 minutes prior to destaining with 150 μ l 95% ethanol. The OD₅₉₅ was measured and the biofilm is quantified as the absorbance normalized to the initial cell density. When cultures were grown for less than 18 hours, the biofilm production was non-existent for the wild-type that measurements were impossible. However, growth of cultures beyond 20 hours caused so much clumped bacterial cells that most were sometimes expelled with the medium, making measurements highly variable.

The quantitative hemolysin assay was adapted from a previously published paper (Fan et al) with modifications (Mahoney et al., 2010). In brief, 10 μ l of the overnight culture was transferred into a fresh polystyrene microplate with 300 μ l of HI media per well and grown at their respective temperature for 2.5 hours, in order for the cells to reach log growth when hemolysins are optimally detected (Mahoney et al., 2010). The OD₆₀₀ was measured for the initial optical density and 95 μ l of culture was combined with 800 μ l of a blood mix containing 40 ml of 1X phosphate-buffered saline (PBS) and 500 μ l of defibrinated sheep's blood (Innovative Research, Novi, MI). Cells were pelleted through centrifugation at 4,000 rpm (3,670Xg) for 10 minutes and incubated for 18 hours at their respective temperatures. The next day pellets were resuspended and the cell debris removed by centrifugation at 4,000 rpm for 10 minutes. The amount of heme present in the cleared cell lysate is determined by the OD₄₁₅ as a measurement of red blood cell lysis and normalized to the initial optical density (OD₆₀₀).

A high-throughput quantitative protease assay was adapted from a standard azocasein procedure (Braun et al., 1980; Prestige et al., 1971). In brief, the initial OD₆₀₀ of overnight cultures were measured and 100 μ l were mixed with 150 μ l of 2% azocasein solution in milliQ water. The inoculated azocasein mixture was incubated for 2.5 hours at the respective temperature of the overnight cultures. After incubation, the reaction was suspended with the addition of 150 μ l of 10% trichloroacetic acid and centrifuged at 4,000 rpm for 15 minutes. One hundred μ l of supernatant was transferred to a new polystyrene microplate. The OD₄₄₀ of the supernatant was determined as a measurement of azocasein cleavage by protease and normalized to the initial optical density.

Gastric virulence assays with *C. elegans*. Mutants were characterized for changes in virulence potential using a recently established nematode model (Chapter 2). The nematodes were prepared using the synchronization of *C. elegans* protocol previously described and the solid substrate gastric virulence assay previously described was performed (Chapter 2). In short, nematode populations were synchronized to the same age and developmental stage by killing of adults with ice-cold bleach solution. The eggs were isolated by centrifugation and washed with M9 to reduce bleach carryover. Eggs were then plated onto 50 ml NGM plates seeded with *E. coli* OP50 as a food source and maintained at room temperature for 3 days.

Capsule mutants were selected for further analysis in nematodes with with MDOH-04-5M732 as the pathogenic positive control reference strain and *E. coli* OP50 as the negative non-pathogenic control. Strains of *V. parahaemolyticus* were grown in HI overnight at both 28°C and 37°C with shaking at 200 RPM, whereas *E. coli* OP50 was grown in LB broth. One-hundred µl of the overnight culture was spread in a circle leaving ~2 cm of clearing to the edge of the plate. Plates were inoculated in triplicates and incubated overnight at their respective temperatures. L4 nematodes were washed off the 50 ml NGM plate with M9 buffer and concentrated through centrifugation. Pelleted nematodes were washed with M9 buffer to limit *E. coli* OP50 carryover. About 25 nematodes in 100 µl of M9 were plated 2 cm away from the bacterial lawn onto each assay plate. Assay plates with overnight cultures grown at 37°C were incubated for 6 hr at 37°C before transfer and overnight growth at 28°C, whereas plates from overnight cultures grown at 28°C were maintained at a constant temperature of 28°C. Mobility rate of infected nematodes was assessed as the time in seconds for 15 individual nematodes to

migrate 2 cm under a 1.5x magnification on a dissecting scope, expressed as the average. Severity of virulence within infected nematodes was assessed based on mobility rate changes compared to the wild-type reference and the significance of the results was analyzed through a one-way ANOVA using the JMP software.

Fluorescence labeling. MDOH-04-5M732 mutants with decreased capsule layer and virulence were fluorescently tagged with GFP(pVSV102) and RFP (pVSV208) plasmids using a triparental mating method (Stabb et al., 2002). Briefly, *V. parahaemolyticus* MDOH-04-5M732 mutants were grown in HI overnight at 28°C. *E. coli* donor for GFP and helper were grown in LB with kanamycin (Km) (50 µg/ml) while *E. coli* donor for RFP was with the addition of chloramphenicol (Ch) (25 µg/ml) overnight at 37°C. Bacterial cultures were centrifuged at 13,500 rpm for 2 minutes and washed with antibiotic-free HI media. Donor, helper, and recipient MDOH-04-5M732 were mixed together and spotted in 100 µl of HI onto an HI agar plate. Mating spots were incubated at 37°C for 4.5 hours and serially diluted onto either a HI Km plate for GFP or HI Ch for RFP. Plates are then incubated at 25°C and colonies that form overnight are visibly screened for fluorescence, isolation streaked, and stored in 27% glycerol at -80°C.

Bacterial competition. To determine whether the decreased virulence of capsule mutants in nematode correlated to a decreased ability to colonization, capsule mutants were competed against the wild-type MDOH-04-5M732 using the bacterial competition procedure previously described (Chapter 2). In short, the OD₆₀₀ of overnight cultures were standardized to a starting optical density of 2.0. Each culture was transferred to a microcentrifuge tube, pelleted, and washed with M9 buffer. Competition strains were mixed, resuspended in HI and spread in a 5 cm diameter circle in the center of NGM

plate. Synchronized nematodes (~50) were washed with M9 and spotted 1 cm above the bacterial lawn. Plates were maintained at room temperature for 24 hours and the colonization of nematodes was viewed under the confocal microscope.

C. elegans fed oppositely fluorescence labeled capsule mutant mixed with MDOH-04-5M732 were washed off the plate and centrifuged at 1,000 rpm for 1 minute. Infected nematodes were washed 5 times with M9 buffer containing 1 mM sodium azide. Approximately 30 nematodes in 100 µl were transferred to a microcentrifuge tube and incubated at -80°C for 15 min to inhibit nematode movement on the confocal. About 20 nematodes were placed onto a slide with the coverslip adhered with the VaLaP sealant. Colonization within infected nematodes was analyzed with the Zeiss software provided with the scanning laser confocal microscope for location and intensity of the fluorescence. The starting bacterial inoculum was calculated by suspension and serial plating of the bacterial lawn onto HI Km and HI Ch.

Bacterial colonization. In addition, about 20 infected nematodes were homogenized as previously described (Chapter 2) using 400 mg of 1.0 mm silicon carbide particles to desiccate the nematodes in 250 µl of M9 buffer containing 1 mM sodium azide. Tubes were vortexed at maximum speed for 1 minute and the resulting homogenate was diluted and plated onto HI media containing Km to determine CFU of the GFP and HI with Ch to determine CFU of the RFP strains

Genetic techniques and analysis. The site of transposon insertion in select mutants with altered virulence was identified by amplification by arbitrary primed PCR method followed by sequencing (Das et al., 2005). Genomic DNA was extracted from detergent-lysed cells using silica columns following manufacturers protocols (Epoch

Biolabs, Inc. 2002). The transposon-genomic DNA junction was amplified in two rounds of PCR using primers shown in Table 3.2. First round reactions contained 5.95 µl nuclease-free water, 1.05 µl 10x buffer, 2.1 µl 5x buffer, 0.16 µl deoxyribonucleotide, 0.21 µl TnERM4 forward primer, 0.53 µl Arb1 reverse primer, and 0.5 µl Taq polymerase. First round conditions required denaturation at 94°C for 30 seconds with the first cycle running for 4 minutes, annealing at 36°C with an additional minute added per cycle, and extension at 72°C for 3 minutes. Reactions were ran for 12 cycles and held at 10°C in the thermocyclers until storage at 4°C. Second round reactions contained 8.83 µl nuclease-free water, 1.65 µl 10x buffer, 3.3 µl 5x buffer, 0.26 deoxyribonucleotide, 0.33 µl 170extb forward primer, 0.33 µl Arb2 reverse primer, and 0.05 µl Taq polymerase. Second round parameters consisted of denaturation at 94°C for 30 seconds, annealing at 65°C for 30 seconds, and extension at 72°C for 3 minutes with a total of 25 cycles. PCR products were ran on a 0.7% agarose gel in 1x TAE at 60 volts for 90 minutes and the presence for sequencing products were examined. After confirmation, the PCR product is sent to the Hubbard Genome Center (University of New Hampshire, Durham, NH) for Sanger sequencing.

RESULTS:

Distribution of virulence associated phenotypes. To facilitate the identification of previously unidentified virulence factors of *V. parahaemolyticus*, and characterize the coordinated regulation of virulence-associated traits, especially in response to human body temperature, we applied random mutagenesis to generate strains altered in potential

or proposed virulence traits. A total of 1,760 mutants were generated in pathogenic *V. parahaemolyticus* strain MDOH-04-5M732 and subsequently screened for alterations in phenotypes in biofilm, siderophore, hemolysis, motility, protease, and extracellular polysaccharide relating to capsule. General auxotrophies were also assessed. Of the 1,760 mutants generated, 576 mutants differed phenotypically from wild-type MDOH-04-5M732 (Fig. 3.1). Of these 576 transposon insertion mutants, 479 insertions resulted in pleiotropy and only 97 mutants were altered in only one observed phenotype. Changes in biofilm and siderophore dominated the 576 mutants. The number of biofilm overproducers and underproducers were similar. Biofilm overproducers had on average 10x more biofilm than the wild-type whereas underproducers had little to no biofilm production. Siderophore mutants tended to produce 10x less than wild-type, and few over-produced siderophore, on average 2x the wild-type level. All 26 auxotroph mutants had a gene disruption that resulted in pleiotropy. Wild-type MDOH-04-5M732 produces no hemolysin at 28°C, however, 71 mutants produced hemolysin at this temperature. Of these, 44 mutants also over produced hemolysin at 37°C, indicating an overall overproduction of hemolysin not specifically related to temperature. Although some of these mutations may reveal general repression mechanism of hemolysin production, some may be due to a response to general stress. A previous mutant identified with a hemolysin overproduction at both 28°C and 37°C had a disruption in the *tolA* gene, a membrane integrity protein, which may result in a leaky membrane due to membrane stress. However, 27 mutants produced hemolysin at 28°C and produced normal levels at 37°C, suggesting the loss of repression at the lower temperature. These mutants may reveal genes involved in temperature regulation. A total of 41 mutants were hemolysin

underproducers at 37°C. Further investigation is needed to identify non-producers from this population since non-producers at 37°C could map to hemolysin genes or regulators that activate hemolysin production at human body temperature.

The individual values from the phenotypic screens were displayed in a heat map to assess hierarchy relationships and phenotypic correlations (Fig 3.1) Based on additional pair-wise comparison between phenotypes, some pleiotropic trends were particularly apparent (Fig 3.2). Mutants that had a decreased biofilm production also often had an increase in siderophore production; however, the inverse of these two phenotypes was not as prominent among the screened mutants. Mutants with decreased capsule production also frequently had an increase in biofilm production.

Capsule mutants are less virulent in the *C. elegans* gastric infection model.

Because mutants of *V. vulnificus* with decreased capsule production are less virulent in mice (Wright et al., 1990), we chose one class of mutants altered only in capsule production for focused analysis in nematodes, and included a few select mutants that produced hemolysin at 28°C for comparison. The nematode gastric infection model was utilized to measure whether reduced capsule in 22 non-pleiotropic mutants alters the virulence *in vivo* measured as changes in mobility rate (distance per second) relative to wild-type MDOH-04-5M732. For reference these assays also included a non-pathogenic control, *E. coli* OP50. The mobility rate increased for all capsule mutants indicating reduced capsule decreased virulence by *V. parahaemolyticus* suggesting a role for capsule in virulence. Nematodes fed mutant JS4D9 had the fastest mobility rate at 28°C with an average mobility rate of 0.13 cm/s while the non-pathogen control had an average mobility rate of 0.19 cm/s. Nematodes fed mutants JS6E3 and JS7B8 had the fastest

mobility rate at 37°C. In contrast, several mutants with altered hemolysin were unaltered in virulence in the same assays (JS1H7 and JS7H9 on Fig 3.3)

Capsule mutants with decreased virulence contain gene disruptions in a variety of cellular functions. The identity of genes disrupted by the transposon insertions for nine of the capsule mutants with reduced virulence were identified (Table 3.3). The disrupted genes for four mutants, including JS4F9, JS6B1, JS6D2, and JS6E3, have deduced functions in metabolic processes that were unlikely to directly contribute to the production of capsule. These genes are responsible for producing cellular products from the fumarate and purine pathways and fatty acids could play an indirect role in the proper formation of the capsule. The involvement of an extracellular toxin, such as alkaline serine protease (JS4F8) and thermostable direct hemolysin S (JS4G9), and the MutT/nudix family (JS4G7) in capsule production is unclear. Although the mechanism of the cold shock regulator CspA in regulating capsule is not well understood, the increased expression of the CspA regulator in *S. pneumoniae* increased the production of capsule (Corbett et al., 2010). The probable loss of function in the *cspA* gene and accompanying decrease in capsule suggests this regulator has similar regulatory function in *V. parahaemolyticus*. Two genes identified in mutants MC27 and JS4E9 are responsible for the synthesis of the enzymes UTP-glucose-1-phosphate uridylyltransferase and mannose phosphate isomerase respectively. Both of these enzymes are involved in the production of capsular polysaccharide precursor molecules.

Capsule mutants were impaired in colonization of nematode intestines. Two capsule mutants, JS4D9 and JS6E3, were further analyzed for changes in their colonization ability within the nematode intestine as well as when competed with the

wild-type in nematode intestine. These 2 mutants were chosen because they were the least virulent capsule mutants in the nematode gastric infection model. These two mutants were competed with MDOH-04-5M732 to determine their changes in colonization ability as a result of their decreased virulence in the nematode gastric model. The mutant and wild-type were fed to nematodes and their colonization within the nematode intestine was assessed after 24 hours under the confocal laser scanning microscope. Both JS4D9 (Fig 3.4A and 3.4B) and JS6E3 (Fig 3.4C and 3.4D) were fluorescently labeled with GFP. These capsule mutants were unable to colonize the nematode intestine and were localized to the pharynx region.

In pair-wise competition with the RFP tagged wild-type MDOH-04-5M732, capsule mutants were unable to colonize in nematode intestine, while MDOH-04-5M732 colonized throughout the gastrointestinal tract (Fig. 3.4E to 3.4H). JS4D9 (Fig 3.4E and 3.4F) and JS6E3 (Fig 3.4G and 3.4H) were localized in the mouth and pharyngeal region of the nematodes. At the start of the competition JS4D9 and MDOH-04-5M732 had similar concentrations; however, the recovery of JS4D9 cells from infected nematodes were 1 log lower than the wild-type (Table 3.4). When JS6E3 was competed with MDOH-04-5M732, the competition result was identical. The competitive index of 4D9 and 6E3 were 0.08 and 0.06 respectively. The disruption of the gene within these mutants greatly reduced their ability to colonize the nematode intestine. These results showed that capsule mutants have decreased virulence in the nematode gastric model and a reduced fitness in colonization of nematode intestine.

DISCUSSION:

In order to gain a better understanding of the virulence-associated traits that contribute to the virulence mechanism of *V. parahaemolyticus*, a library of 1,760 transposon mutants was screened for alterations in phenotypes that potentially contribute to virulence. The virulence-associated traits included biofilm formation, siderophore, hemolysis, motility, protease, and capsule have all been implicated for playing a role in pathogen virulence (Caburlotto et al., 2010). Thus, changes in these phenotypes may influence the virulence potential of pathogens during infections. Additionally, hemolysin, motility, and protease are positively regulated in response to the human body temperature with increased expression on phenotypic assays (Mahoney et al., 2010), and therefore were assessed at both 28°C and 37°C to potentially identify regulators of coordinated gene expression during host infection. Temperature regulation of virulence may provide a novel method to distinguish pathogens from harmless environmental strains. A total of 576 mutants were identified containing a gene disruptions affecting the phenotypes screened when compared to wild-type. Even though mutants were generated through multiple mating events, the presence of redundant or sister mutants cannot be excluded. Out of the 576 mutants, only 97 mutants contain a mutation that did not induce a pleiotropic effect. Pleiotropic mutants may have arisen due to a insertion of the transposon into an operon causing downstream expression or transposon insertion caused a polar effect on surrounding genes.

Non-pleiotropic mutants with decreased production in capsule were chosen for further analysis due to the importance of capsule production in the closely related species, *V. vulnificus* where capsule production is a prerequisite for virulence and

correlates to lethality in mice (Wright et al., 1990). Here we show capsule production is also crucial in the colonization of *C. elegans* intestine by *V. parahaemolyticus*, and decreased capsule reduced fitness as assessed by direct competition with the wild-type MDOH-04-5M732 (Fig 3.4). Capsule mutants were localized to the pharynx region and unable to proliferate in the intestinal tract. Capsule mutants had decreased virulence and colonization in nematodes. The fact that mutants with altered hemolysin were unaltered in virulence suggests that the presence of the transposon alone did not decrease virulence and that a phenotype specifically associated with the insertions reduced virulence. Although without a targeted mutation specific to capsule synthesis the possibility remains that other phenotypes associated with the mutation the observation that all capsule mutants had impaired virulence based on increased nematode mobility rate provides compelling evidence for the role of capsule in virulence. Further assessment with the use of MC27 and JS4E9 which are mutants with a disruption in genes directly involved in capsule production would support these conclusions. Production of capsule is necessary for resistance to phagocytosis and complement-mediated lysis (Wright et al 1990). Thus, the lack of colonization observed in nematodes may be attributed to these mutants being more susceptible to host immune defense than the wild-type.

Without screening the entire library in an infection model, virulence factors that do not correlate to an obvious biochemical defect will not be identified. This method of identifying virulence factors requires the availability of an infection model that can be used to screen at a high-throughput rate for alterations in virulence potential. In *P. aeruginosa*, there were some virulence factors that exhibited no detectable defect when disrupted (Rahme et al., 1999). In addition, screening the library through biochemical

defects requires prior knowledge of its virulence relation as well as a developed assay capable of detecting these defects. Currently there are no infection models for *V. parahaemolyticus* available with the capability of screening thousands of mutants efficiently. Condition that promotes the susceptibility of nematodes during *V. parahaemolyticus* infection would be necessary in developing this successful alternative host model into a high-throughput model.

Table 3.1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description ^a	Source or reference
<i>E. coli</i> strains		
DH5α	<i>FrecA₁ endA₁ hsdR₁₇ supE₄₄ thi-1 gyrA₉₆ relA₁Δ(argF-lacZYA) U169 φ80lacZΔM₁₅ λ⁻</i>	Gibco-Bro, Inc.
<i>V. parahaemolyticus</i> strains		
MDOH-04-5M732	Pandemic clinical isolate from NY	Davis et al
Plasmids		
pEVS104	R6Kγ derivative of pRK ₂₀₁₃ ; ΔColE ₁ , <i>oriT tra trb</i> ΔTn ₉₀₃ Km ^r	Stabb et al
pEVS170	mini-Tn5-Erm ^r , <i>oriVR6K_ oriTRP4</i> Knr	E. Stabb

^aErm, erythromycin; Km, kanamycin

Table 3.2. Primers used for arbitrary primed PCR.

Arb 1 Reaction Primers			
		5'	3'
Arb 1	Reverse	GGCCACGCGTCGACTAGTACNNNNNNNNNN	
TnErm4	Forward	AATGCCCTTTACCTGTTCC	
Arb 2 Reaction Primers			
		5'	3'
Arb 2	Reverse	GGCCACGCGTCGAC	
170 Extb	Forward	CGCACTGAGAAGCCCTTAGAGC	

Table 3.3. Genotype of MDOH-04-5M732 mutants with decreased capsule expression.

Strain	Gene locus	Annotation/probable function
<i>DNA binding or repair</i>		
JS4G7	VP2920	MutT/nudix family
<i>Metabolism</i>		
MC27	VP0237	UTP-glucose-1-phosphate uridylyltransferase
JS4E9	VPA1425	mannose phosphate isomerase
JS4F9	VP2289	Oxidoreductase, acyl-CoA dehydrogenase family
JS6B1	VP2840	Fumarate reductase, flavoprotein subunit
JS6D2	VPA1121	Putative acyl-CoA dehydrogenase
JS6E3	VPA1475	Purine nucleoside phosphorylase
<i>Secretion</i>		
JS5A10	VP1408	Putative IcmF-related protein
JS4F8	VPA0227	Alkaline serine protease
JS4G8	VPA1378	Thermostable direct hemolysin S
<i>Regulation</i>		
JS4D9	VP1889	Cold shock transcriptional regulator CspA

Table 3.4. *In vivo* competition assays of wild-type and MDOH-04-5M732 mutants of *V. parahaemolyticus*.

Starting		Competitive Index			Ratio	CI ^a
		Ratio	Ending			
4D9 (GFP) x MDOH-04-5M732 (RFP)						
8.3x10 ⁹	7.9x10 ⁹	1.05	4.2x10 ³	5.3x10 ⁴	7.9x10 ⁻²	0.08
6E3 (GFP) x MDOH-04-5M732 (RFP)						
6.4x10 ⁹	6x10 ⁹	1.07	3.7x10 ³	6.2x10 ⁴	5.97x10 ⁻²	0.06

^aStrains were labeled in reciprocal fluorescence and no differences were observed.

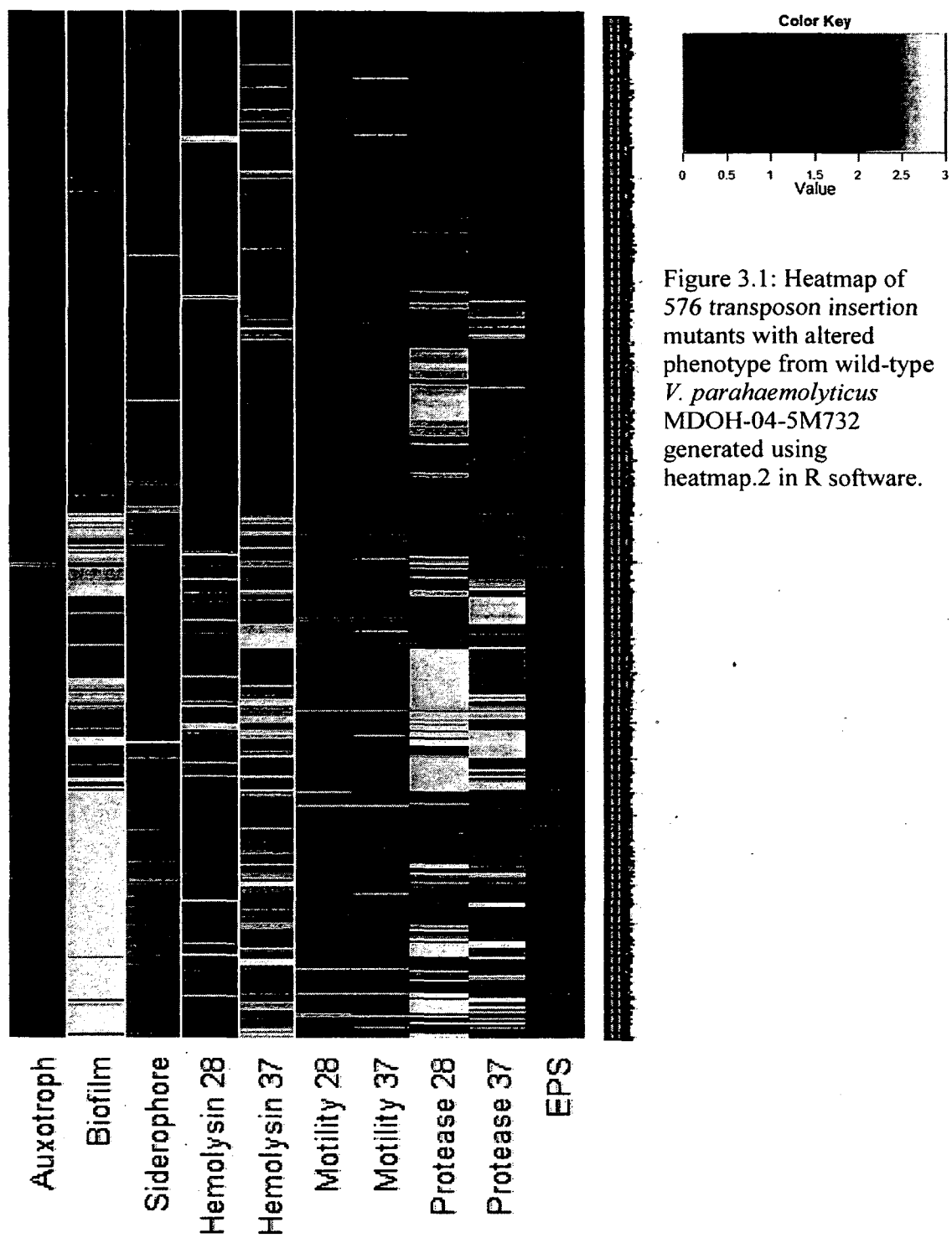


Figure 3.1: Heatmap of 576 transposon insertion mutants with altered phenotype from wild-type *V. parahaemolyticus* MDOH-04-5M732 generated using heatmap.2 in R software.

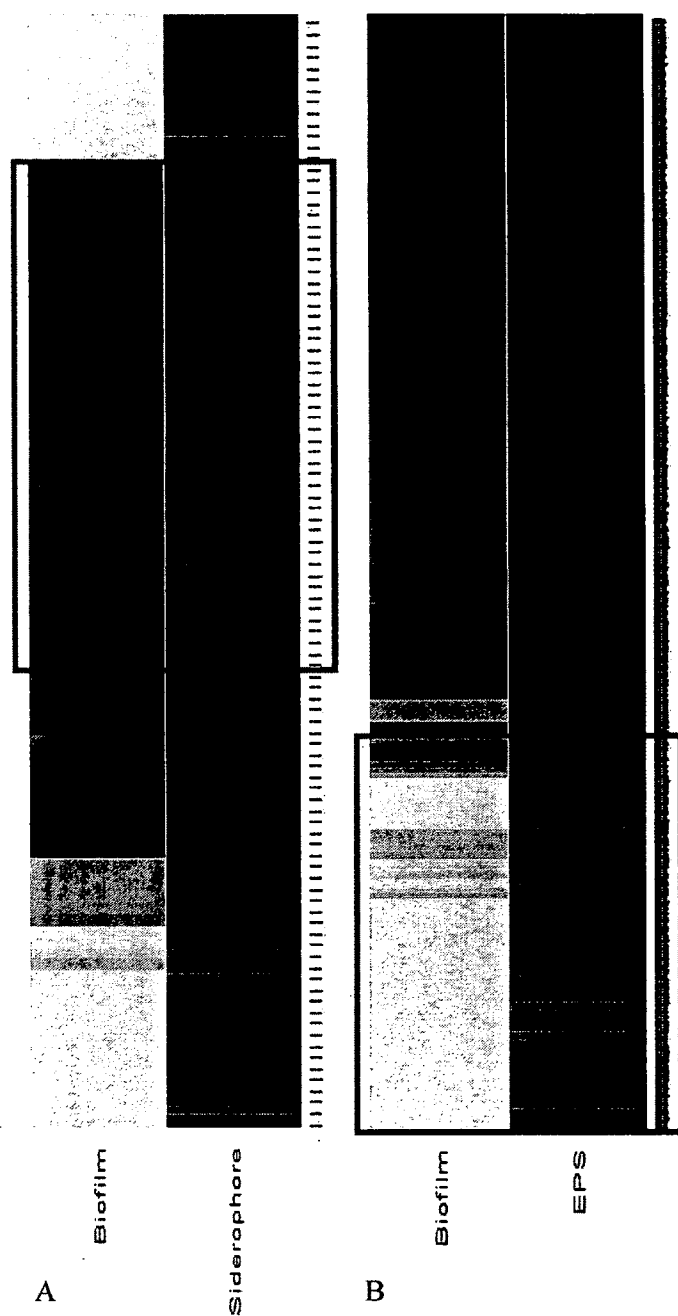


Figure 3.2: Pair-wise comparison of phenotypes in 576 MDOH-04-5M732 transposon insertion mutants. (A) The comparison of biofilm and siderophore mutants with a high correlation between decreased biofilm production and enhanced siderophore production (marked in red). (B) Pair-wise comparison of biofilm and EPS mutants have a high correlation between decreased EPS production and increased biofilm production (marked in red).

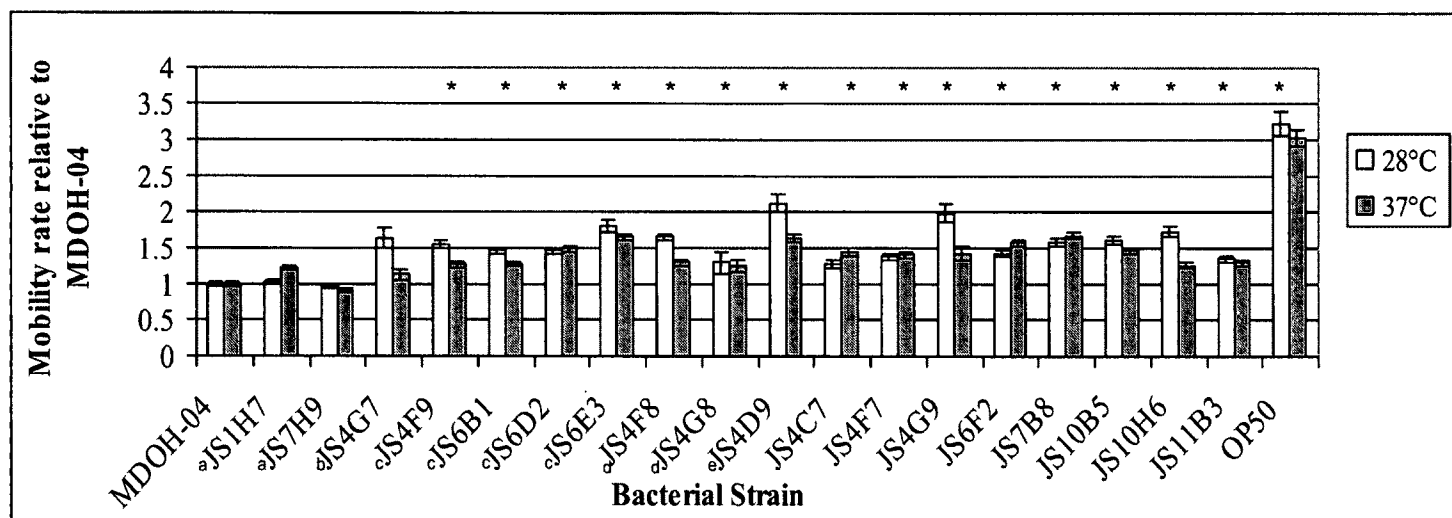


Figure 3.3. *C. elegans* mobility rate fed the MDOH-04-5M732 transposon insertion capsule mutants relative to wild-type. Experiment was conducted at both 28°C and 37°C and *C. elegans* mobility rate was normalized to wild-type MDOH-04-5M732. Error bars show 95% confidence interval and significant differences from wild-type MDOH-04-5M732 at both temperature (*) was determined by one-way ANOVA (Dunnett's with wild-type control). ^aMutants with gene functioning in DNA binding or repair. ^bMutants with gene functioning in metabolism. ^cMutants with gene functioning in secretion. ^dMutants with gene functioning in regulation.

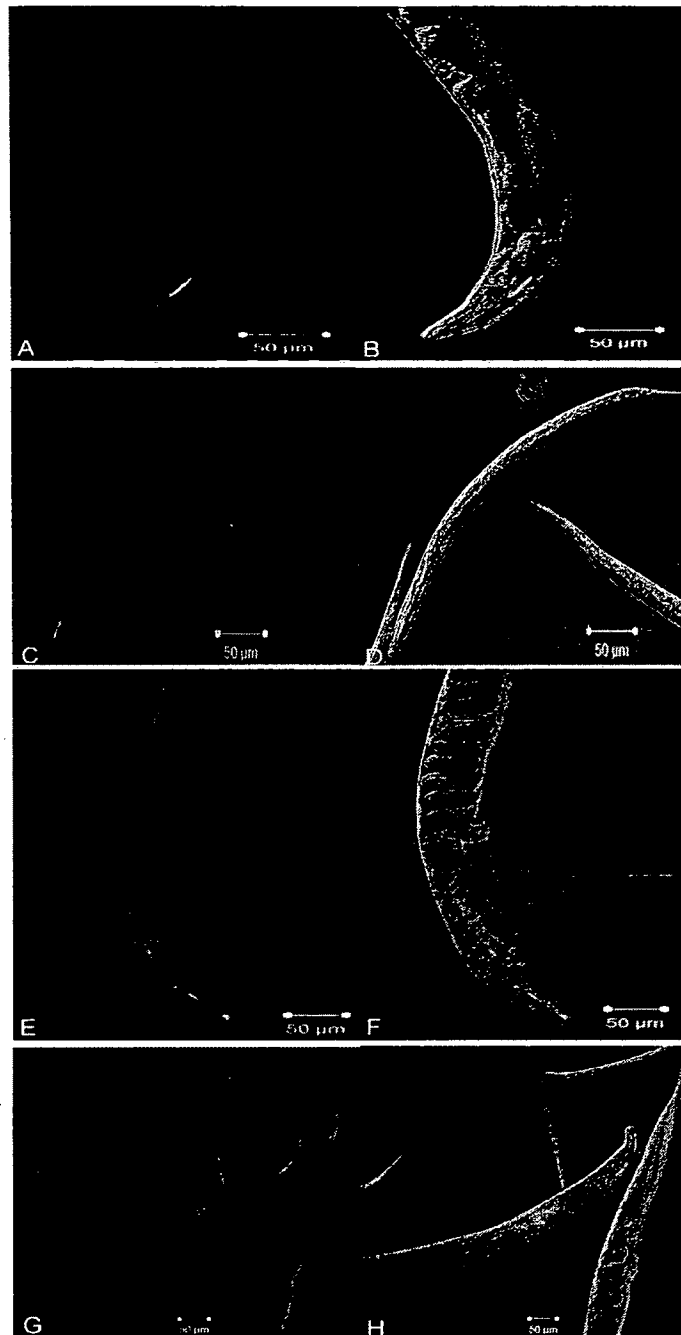


Figure 3.4. Colonization of capsule mutants and pair-wise colonization competition with wild-type MDOH-04-5M732. Colonization of GFP tagged mutants JS4D9 (A and B) and JS6E3 (C and D) were assessed under the confocal laser scanning microscopy. GFP tagged JS4D9 competed with RFP tagged MDOH-04-5M732 showed a lack of colonization of the capsule mutant within the intestinal tract of infected nematodes (E and F). The pair-wise competition with JS6E3 showed a similar result with MDOH-04-5M732 colonizing the entire length of the intestinal tract (G and H).

Chapter IV

Effects of Human Norepinephrine on *Vibrio parahaemolyticus* Virulence in *Caenorhabditis elegans*

ABSTRACT:

Although the nematode *C. elegans* has systematic differences from the human host, chemical manipulation during infection to emulate conditions present in the human gut may improve the ability of this invertebrate to display symptoms of infectious disease. For example, prior work indicates that the exposure of *V. parahaemolyticus* to norepinephrine results in increased bacterial growth as well as cytotoxicity in Caco-2 cells and enterotoxicity in a rabbit ileal loop, suggesting this may be a host cue that activates virulence. Unfortunately, treatment of nematodes with norepinephrine was fatal. However pre-culturing of bacteria in norepinephrine enhanced the virulence of several clinical strains, but not an environmental isolate from NH, within the *C. elegans* gastric model, measured by nematode mobility rate. Surprisingly, nematodes fed an attenuated pathogenic strain of *V. parahaemolyticus* BB22, a GacA⁻ mutant, pre-grown in norepinephrine was the same as nematodes fed the wild-type strain. This treatment did not alter the GacA⁻ mutant's defective phenotypes in siderophore, capsule, and biofilm production or its enhanced hemolytic activity in culture. Additionally, norepinephrine exposure of GacA⁻ to restored normal colonization ability, in this instance by decreasing colonization of the *C. elegans* intestine when in direct competition with BB22. Thus, the virulence of pathogenic *V. parahaemolyticus* strains exposed to norepinephrine is

enhanced in *C. elegans* and addition of hormones can further emulate disease in this alternative model.

INTRODUCTION:

Although environmental parameters, such as temperature, pH, salinity, and osmolarity, influence the growth of bacteria and their virulence gene expression (DePaola et al., 2003), conditions specific to the host environment will likely play an important role in disease. The study of the effect of host signaling molecules on bacteria has become more apparent and investigated, leading to the concept of microbial endocrinology (Freestone et al., 2000). This emerging science proposes that infectious organisms utilize hormones present within the host as environmental cues to initiate growth and pathogenic processes (Lyte et al., 1993). Norepinephrine modulates the ability of enterohemorrhagic *Escherichia coli* to adhere to the colonic mucosa and stimulates the invasion of porcine jejunal explants by *Salmonella* (Chen et al., 2003; Nakano et al., 2007). The catecholamine norepinephrine enhances the growth of *V. parahaemolyticus* and *V. mimicus* (Nakano et al., 2006). Furthermore, norepinephrine increases cytotoxicity of *V. parahaemolyticus* towards Caco-2 cells and enterotoxicity in the rabbit ileal loop (Nakano et al., 2007).

The norepinephrine hormone is a neurotransmitter found in the central nervous system and located within the human gut (Nakano et al., 2006; Nakano et al., 2007). The gastrointestinal tract is responsible for producing over 50% of the norepinephrine found in the human body (Nakano et al., 2007). This hormone influences the virulence of many

gut pathogens (Nakano et al., 2006); however, within the body, it is normally responsible for modulating the functions of the gut, such as smooth muscle contractibility, the submucosal blood flow, and active transepithelial ion transport. These regulations are maintained through the interactions with α - and β -adrenergic receptors (Nakano et al., 2007). Severe tissue injury causes the release of norepinephrine into the peripheral circulation because of the destruction of noradrenergic neurons innervating the traumatized tissue. Conditions that are associated with elevated norepinephrine, including stress exposure, have been reported to increase host susceptibility to disease (Chen et al., 2003).

Since the presence of norepinephrine induces virulence expression of pathogens, this hormone can be used to further humanize the conditions within *C. elegans* infections. This alternative model has proven useful as a surrogate model for *V. parahaemolyticus* infections (Chapter 2). Although this model is not the normal host for *V. parahaemolyticus*, studies in other pathogens reveal virulence factors that are universally required for the establishment of infection in diverse hosts (Hendrickson et al., 2001; Tan et al., 1999). By synthesizing controlled conditions that could act as environmental cues, infections and disease progression may be more relevant to human hosts. These cues, such as temperature and endocrine hormones, will allow some emulation of the human conditions within alternative infection models. Microbe-host interaction can play an important role in enhancing the pathogenesis of pathogenic *V. parahaemolyticus* strains and the use of this human hormone can lead to a better understanding of the disease mechanism within this pathogen.

MATERIALS AND METHODS:

Bacterial strains and culture conditions. *C. elegans* N2 was obtained from the *Caenorhabditis* Genetic Center, Minneapolis, MN. Ten bacterial strains (Table 2.1) were utilized with three positive controls, *B. cenocepacia* HI2424, *P. aeruginosa* PA01, and *V. cholerae* C6706, were included since they are proven pathogens able to infect diverse host lineages such as fruit flies, nematode, and plants (Blow et al., 2005; Cooper et al., 2009; Henderickson et al., 2001; Rahme et al., 1999). The negative reference strain *E. coli* OP50 is a non-pathogenic food source of nematodes (Cooper et al., 2009). In addition, six strains of *V. parahaemolyticus* were chosen to assess the ability of norepinephrine to enhance virulence in a variety of pathogenic and environmental strains (Table 2.1). *V. parahaemolyticus* and *V. cholerae* were grown in heart infusion (HI) broth at a pH of 7.3 (Fluka, Buchs, Switzerland) with shaking at 200rpm at both 28°C and 37°C. *B. cenocepacia* HI2424, *P. aeruginosa* PA01, and *E. coli* OP50 were grown in Luria-Bertani (LB) broth (Sambrook et al., 1989) at both temperatures for assays and at 37°C otherwise. Norepinephrine was supplemented into growth media as needed at a concentration of 50 µmol/L.

Synchronization of *C. elegans*. In order to synchronize the nematode populations to a common developmental stage, a standard bleaching method previously described was applied (Chapter 2). Briefly, nematode populations on NGM agar plates were removed by the addition of ice-cold M9 buffer. The nematodes suspended in M9 were collected into a conical tube with the addition of ice-cold bleach solution which contains 2 ml of household bleach and 1 ml of 5 M sodium hydroxide. The tubes were incubated in ice with 10 second vortexing at maximum speed every 2 minutes for 10 minutes. Nematode

eggs were concentrated by centrifugation and supernatant was removed by leaving ~1 ml to avoid disruption of pellet. Egg pellets were resuspended with ice-cold M9 and washed twice to minimize the carryover of bleach. The eggs were plates onto a fresh NGM plate seeded with *E. coli* OP50 and maintained at room temperature for approximately 3 days for the development of L4 nematodes.

Solid substrate gastric virulence assays with *C. elegans*. Bacterial virulence assays of nematodes on solid agar were performed as previously described (Chapter 2). See appendix for a detailed Standard Operating Protocol. Overnight cultures of each strain (Table 2) were grown with shaking at 200 RPM at either 28°C or 37°C with and without norepinephrine. One-hundred µl of culture was spread as a 5 cm diameter circle in the center of each of three NGM plates, and grown for 24 hours at the same temperature as overnight culturing. Nematodes at the L4 developmental stage in M9 buffer was collected and isolated by centrifugation. Nematodes were washed three times with M9 to minimize bacterial carryover. Approximately 25 L4 nematodes suspended in 100 µl were transferred to each bacterial lawn and incubated at their respective temperatures. Assay plates incubated at 37°C were transferred to 28°C after 6 hours to minimize adverse effects on nematodes. Rate of mortality of nematodes was observed and recorded at 24 and 48 hours. Mobility rates of nematodes from various control and treatment conditions were also recorded on living nematodes by recording the time in seconds required for each of 15 nematodes per treatment to migrate 2 cm under a 1.5x magnification on the dissecting microscope. Significant differences in mobility rates were analyzed using a one-way ANOVA with the JMP software suite (version 10, SAS Institute Inc.).

Phenotyping of GacA⁻ mutant exposed to norepinephrine. The capsule production of the GacA⁻ mutant was screened on 50 ml congo red agar plates as previously described (Chapter 3). The congo red plate was inoculated with 10 µl of each overnight isolate in triplicate. The assay was conducted at 28°C and observed at 24, 48, and 72 hours of growth for variations in pigment uptake. The quantitative siderophore assay was performed to determine the ability of GacA⁻ to produce the iron acquisition chelators. The chrome azurol S standard protocol was performed with slight modifications as previously described (Chapter 3). The quantitative hemolysin assay (Fan et al., 2001) and the quantitative biofilm assay (O'Toole et al., 1999) were performed as previously described (Chapter 3) to determine hemolytic activity and biofilm production of the GacA⁻ mutant respectively.

Fluorescence labeling. *V. parahaemolyticus* BB22 and the attenuated BB22 mutant GacA⁻ was labeled with plasmids expressing GFP (pVSV102) or RFP (pVSV208) through conjugation as previously described (Stabb et al., 2002; Chapter 2). In brief, recipient *V. parahaemolyticus* strains were grown in HI medium for 18 hours with the donor and helper *E.coli* grown in LB medium with the addition of kanamycin (Km) for GFP mating and chloramphenicol (Ch) for RFP mating. Bacterial cultures were centrifuged at 13,500 rpm and pellets were washed with antibiotic-free HI medium. Pellets were resuspended, mixed, and spotted onto HI media. Mating spots incubated at 37°C for 4.5 hours and then serially diluted with HI medium and plated onto HI medium with Km or Ch. Colonies formed after an overnight incubation was visibly screened for GFP or RFP expression under an inverted fluorescent microscope and grown in HI with appropriate antibiotic overnight at 28°C and stored in -80°C for future experiments.

Bacterial competition. *V. parahaemolyticus* BB22 and the attenuated mutant GacA⁻ were reciprocally tagged with GFP or RFP and competed in a pair-wise colonization assay previously described (Chapter 2). Briefly, fluorescently labeled BB22 and GacA⁻ with and without norepinephrine exposure were competed to determine changes in colonization ability. Competing strains were grown overnight, mixed, resuspended in 100 µl of HI media, and spread plated onto a NGM plate. Cultures were spread in a 5 cm diameter circle in the center of the agar plate. Synchronized L4 nematodes (~50) were washed 3 times with M9 buffer and plated 1 cm above the bacterial lawn. Plates were incubated at room temperature for 24 hours and viewed under the confocal microscope as well as homogenized to determine the colony forming unit of colonized bacteria.

C. elegans from the competition assays were prepared for microscopic observations as previously described (Chapter 2). Briefly, infected nematodes were washed with M9 buffer containing 1 mM sodium azide. Approximately 30 nematodes were transferred to a microcentrifuge tube and placed in the -80°C for 15 minutes in order to kill the nematodes without damaging the bacterial fluorescence. 20 nematodes were placed onto a slide and the coverslip sealed with VaLaP sealant and the localization of bacteria within *C. elegans* intestine was analyzed with the Zeiss software provided with the confocal laser scanning microscope. The starting bacterial inoculum was calculated by washing the bacterial lawn off plates with 2 ml M9 buffer, serially diluting, and plating onto HI media containing the appropriate antibiotics and incubating at 28°C. The colony forming unit (CFU) of both the GFP and RFP labeled strains was calculated to determine the bacterial concentration nematodes were exposed to.

Bacterial colonization. The bacterial colonization of nematodes was quantified from a standard protocol previously described (Chapter 2). In short, approximately 20 infected nematodes were obtained from the competition assay in addition to washing the bacterial lawn with 2 ml of M9 with 1 mM sodium azide. Nematode suspension was transferred to a microcentrifuge tube containing 400 mg of 1.0 mm silicon carbide particles (Biospec, Bartlesville, OK). The total volume was brought up to 250 μ l with M9 buffer containing 1 mM sodium azide and tubes were vortexed at maximum speed for 1 minute to homogenize the nematodes without affecting bacterial survival. The resulting homogenate was diluted and plated onto HI with Km and HI with Ch to calculate the CFU of GFP strain and RFP strain respectively. The competitive index was calculated based on a standard protocol (Mey et al., 2002) by using the ratio of mutant to wild-type bacteria recovered from the intestine of 20 nematodes were determined and then normalized by dividing by the ratio of mutant to wild-type bacteria in the initial inoculum.

RESULTS:

Norepinephrine enhanced bacterial virulence of pathogenic strains and *E. coli* OP50 in the *C. elegans* gastric infection model. The human hormone norepinephrine, normally found within the gastrointestinal tract, influences the growth of many pathogenic bacteria (Nakano et al., 2006). The addition of norepinephrine in growth media stimulated the growth of *V. parahaemolyticus* and *V. mimicus* but not *V. cholerae* and *V. vulnificus* (Nakano et al., 2006). Furthermore, the addition of

norepinephrine increased cytotoxicity in human Caco-2 cells and enterotoxicity in the rabbit ileal loop model (Nakano et al., 2007). These past studies focused specifically on the effect of norepinephrine on the pathogenic strains of *V. parahaemolyticus* and did not include non-pathogenic strains.

For our evaluation on the effect of norepinephrine, we included the same 10 bacterial strains of pathogens and non-pathogens as previously described (Chapter 2) (See methods for detail). Norepinephrine was added to the bacterial growth media and the influence of this pre-treatment on the mobility rate of nematodes infected with bacteria was assessed at both 28°C and 37°C compared to untreated bacteria (Fig 4.1). Upon inclusion of norepinephrine, nematode mobility rate of living nematodes fed the positive controls *B. cenocepacia* HI2424 and *V. cholerae* C6707 significantly decreased, whereas mobility of nematodes colonized with *P. aeruginosa* PA01 did not. The addition of norepinephrine to the negative control *E. coli* OP50 also significantly decreased the mobility rate of nematodes fed the negative control. The addition of norepinephrine to the pathogenic and attenuated *V. parahaemolyticus* strains caused a significant decrease in the mobility rate of nematodes fed these strains. Due to the death of nematodes at 37°C, the effect of norepinephrine on mobility rate of nematodes fed the positive pathogen controls (i.e. *P. aeruginosa* PA01 and *V. cholerae* C6706) could not be assessed; however, this hormone did significantly reduce the mobility rate of nematodes fed the non-pathogenic negative control, *E. coli* OP50. The addition of norepinephrine caused a significant decrease in mobility rate of nematodes fed the pathogenic *V. parahaemolyticus* strains but did not alter the mobility rate of nematodes fed the environmental strain G61. Thus, the addition of norepinephrine resulted in an increased

bacterial virulence only by pathogenic strains of *V. parahaemolyticus* and not the representative environmental isolate.

The addition of norepinephrine in growth media restored virulence to the GacA⁻ mutant. Unexpectedly, the mobility rate of nematodes fed the GacA⁻ strain, an attenuated mutant of the BB22 as shown in mice (Mahoney et al., 2011), had the same mobility rate of nematodes fed the wild-type BB22 with the addition of norepinephrine (Fig 4.1). There was no significant difference between the mobility rate of nematodes fed the GacA⁻ or BB22 strain grown in norepinephrine. However, the mobility rate of nematodes fed the GacA⁻ strain without the addition of norepinephrine was significantly faster than nematodes fed the wild-type BB22. Thus the addition of norepinephrine restored the virulence of GacA⁻ to wild-type levels.

***In Vitro* phenotypes of the GacA⁻ mutant were not restored by addition of norepinephrine.** The GacA⁻ strain contains a mutation in the conserved global regulator known as GacA⁻, which regulates virulence and host association in many bacterial species. The *V. parahaemolyticus* GacA⁻ strain has a higher hemolytic activity but is defective in several traits, such as siderophore, biofilm, capsule, and metabolism (Mahoney et al., 2011). Functional assays of virulence such as cytotoxicity against human colon cells and disease in mice showed that GacA⁻ had a defect in both systems (Mahoney et al., 2011).

Since the GacA⁻ strain is less pathogenic in multiple models, the restoration of its virulence with the addition of norepinephrine was unexpected. The GacA⁻ mutant is defective in many phenotypes that are believed to contribute to virulence so possible restoration of its *in vitro* phenotypes was evaluated. The GacA⁻ mutant was screened for

changes using both quantitative assays, such as hemolysin and biofilm production, and qualitative assays, such as the CAS agar for siderophore production and Congo red plates for capsule production. GacA⁻ exposed to norepinephrine had no significant difference in the production of hemolysin compared to GacA⁻ without exposure to norepinephrine. In both cases, the GacA⁻ mutant expressed, on average, 1.5x more hemolysin than BB22. The GacA⁻ mutant exposed to norepinephrine still had defects in biofilm, siderophore, and capsule production. Thus, the GacA⁻ mutant's restored virulence in *C. elegans* when exposed to norepinephrine was not due to the restoration of its defective phenotypes.

The enhanced fitness of GacA⁻ in nematode intestines during competition with BB22 decreased with the exposure to norepinephrine. Though defective at initiating disease, the GacA⁻ mutant had enhanced fitness in a mouse intestine when compared to wild-type in a competition assay (Mahoney et al., 2011). In that study, there was a 3-fold increased survival of the GacA⁻ mutant over wild-type based on the % CFU that survived after 1 hour.

The colonization of the GacA⁻ strain fluorescently labeled with RFP was competed against the wild-type BB22 labeled with GFP with and without the addition of norepinephrine. Fluorescent images of infected nematodes were taken after 24 hours of exposure. The localization of both GacA⁻ and BB22 were assessed in nematode intestine scanning laser confocal microscopy and the competitive index was calculated from the starting and ending CFUs and ratios.

In absence of norepinephrine, the GacA⁻ mutant was visible throughout the entire intestinal tract of infected nematodes when competed with BB22 (Fig 4.2A and 4.2B); whereas BB22 was rarely visible even though the starting ratio of BB22 was 2 times

higher (Table 4.1). With the addition of norepinephrine, the GacA⁻ mutant no longer fully colonized the intestine and appeared similar to BB22 (Fig 4.2C and 4.2D). BB22 colonized infected nematodes sporadically along the entire length of the intestinal tract. When both GacA⁻ and BB22 were grown in the presence of norepinephrine, the GacA⁻ mutant was also no longer dominant in the nematode intestine (Fig 4.2E and 4.2F). The competitive index of GacA⁻ decreased from 2.89 to 1.88 when exposed to norepinephrine. This decrease in GacA⁻'s competitiveness against BB22 was greater when both strains were exposed to norepinephrine. Based on the decrease in competitive index of the GacA⁻ mutant, norepinephrine decreased its ability to colonize the nematode intestine, even though it increased its virulence.

DISCUSSION:

Many bacterial pathogens regulate the expression of virulence factors in response to changes in the environment. For example, the level of virulence gene expression in *Listeria monocytogenes* is dependent on the amounts of the PrfA protein, which is expressed at 37°C (Sahu et al., 2012). This temperature-dependent virulence expression was also observed in *Shigella* species (Maurelli et al., 1984). *V. cholerae* virulence genes are coordinately regulated by external stimuli, such as temperature, pH, and osmolarity. The hemolytic activity of El Tor *V. cholerae* was reported to reach a maximum faster with increasing temperature from 22°C to 35°C; meanwhile the rate of decay of activity was significantly retarded at lower temperatures (Sahu et al., 2012). The human hormone,

norepinephrine, promotes the virulence of *V. parahaemolyticus* and may act as a host cue by this pathogen to cause infection (Nakano et al., 2006; Nakano et al., 2007).

In this study, the effect of norepinephrine on virulence was assessed in both environmental and pathogenic *V. parahaemolyticus* strains. Temperature shift did elicit different responses in virulence among the clinical and environmental isolates, thus this human hormone may help solidify the distinction between these groups (Mahoney et al., 2010). Previous studies focused on the effect of norepinephrine on the virulence of clinical strains and more specifically through the association with the T3SS (Nakano et al., 2007). In this study, the addition of norepinephrine enhanced virulence of pathogenic strains and *E. coli* OP50, whereas it did not increase virulence by G61 (Fig 4.1). Norepinephrine increased *V. parahaemolyticus* enterotoxicity in the rabbit ileal loop through interactions with α_1 -adrenergic receptors (Nakano et al., 2007). Thus, the lack of virulence enhancement by norepinephrine within the environmental strain may be due to the lack of a T3SS2 which contributes to enterotoxicity during infection (Park et al., 2004; Pineryo et al., 2010). Interestingly, the virulence of the GacA⁻ mutant, which is attenuated in disease in both mice (Mahoney et al., 2011) and *C. elegans* (Chapter 2), was restored to wild-type levels. Restoration of virulence in the GacA⁻ mutant may mean that interaction with this hormone induces a significant increase in enterotoxicity either by the activation of T3SS2 expression or increased expression of the effectors. Further investigation into this mechanism may allude to important interactions between a pathogen and the host during infection.

To determine whether the virulence restoration of the GacA⁻ strain was attributed to a restoration in virulence-associated traits or some unknown mechanism, phenotypic

assays were performed. The GacA⁻ mutant is defective in siderophore production, biofilm formation, and capsule production; as well as an overproduction of hemolysin (Mahoney et al., 2011). From the phenotypic assays, it was seen that biofilm formation, hemolysin production, siderophore production and capsule production of GacA⁻ were not significantly altered when exposed to this hormonal cue. This may mean that the enhanced virulence in GacA⁻ is due to interaction of this hormone with other virulence pathways.

Although defective in initiating disease, the GacA⁻ mutant was shown to have enhanced fitness in colonization of *C. elegans* intestine. These findings were similar to previous studies of competitions within mouse intestines (Mahoney et al., 2011). The enhanced survival of the GacA⁻ mutant may be attributed to the lack of gastroenteritis seen during infection, which may allow it to better evade immune responses due to a lack of immune activation. In previous studies, GacA⁻ was more resistant to complement-mediated killing than wild-type (Mahoney et al., 2011). This would explain the higher recovery of GacA⁻ from nematode intestines since the wild-type cells were more susceptible to immune defenses. Interestingly, when GacA⁻ was exposed to norepinephrine, its selective advantage in competition with wild-type decreased and when wild-type was also exposed to norepinephrine, GacA⁻ lost its fitness advantage. Since the phenotype of GacA⁻ was not restored with exposure to this hormone, the decreased fitness and recovery of cells may be attributed to a decrease in immune evasion. GacA⁻ had a more potent infection with the presence of norepinephrine, which may cause it to trigger a greater amount of host immune defense. Although at the early

stages, this may demonstrate an important microbe-host interaction and further analysis of pathogen and host responses is required to elucidate this important interaction.

Table 4.1. *In vivo* competition assays of wild-type and GacA⁻ mutant of *V. parahaemolyticus*.

Starting	Competitive		Index		Ratio	CI ^a
	Ratio	Ending	Ratio	Ending		
GacA ⁻ (RFP) x BB22(GFP)	1.7x10 ⁹	3.8x10 ⁹	4.47x10 ⁻¹	6.7x10 ⁴	5.2x10 ⁴	2.89
GacA ⁻ w/NE (RFP) x BB22 (GFP)	4.1x10 ⁹	5.6x10 ⁹	7.32x10 ⁻¹	4.4x10 ⁴	3.2x10 ⁴	1.88
GacA ⁻ w/NE (RFP) x BB22 w/NE (GFP)	1.03x10 ¹⁰	5.3x10 ⁹	1.94	1.26x10 ⁵	1.03x10 ⁵	0.63

^aStrains were labeled in reciprocal fluorescence and no differences were observed.

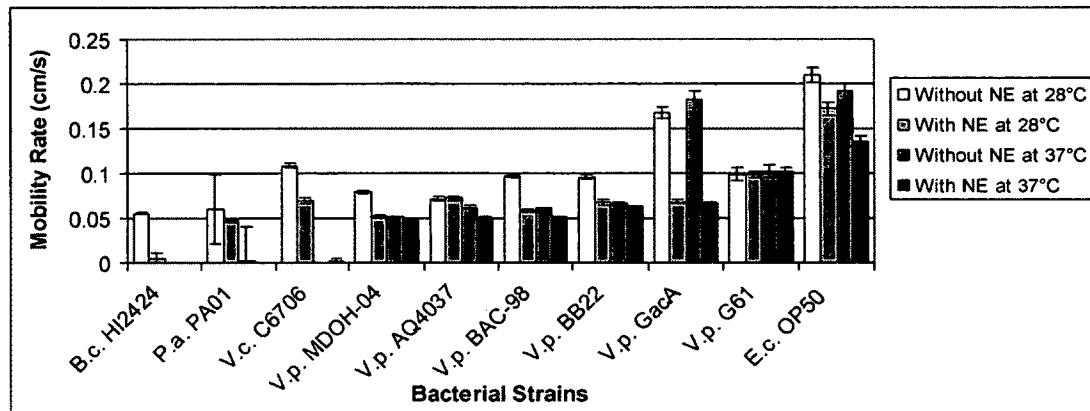


Figure 4.1. Mobility rate of *C. elegans* fed bacteria with and without exposure to norepinephrine at both 28°C and 37°C. No bars indicate death of the nematodes and error bars show 95% confidence interval between nematodes scored.

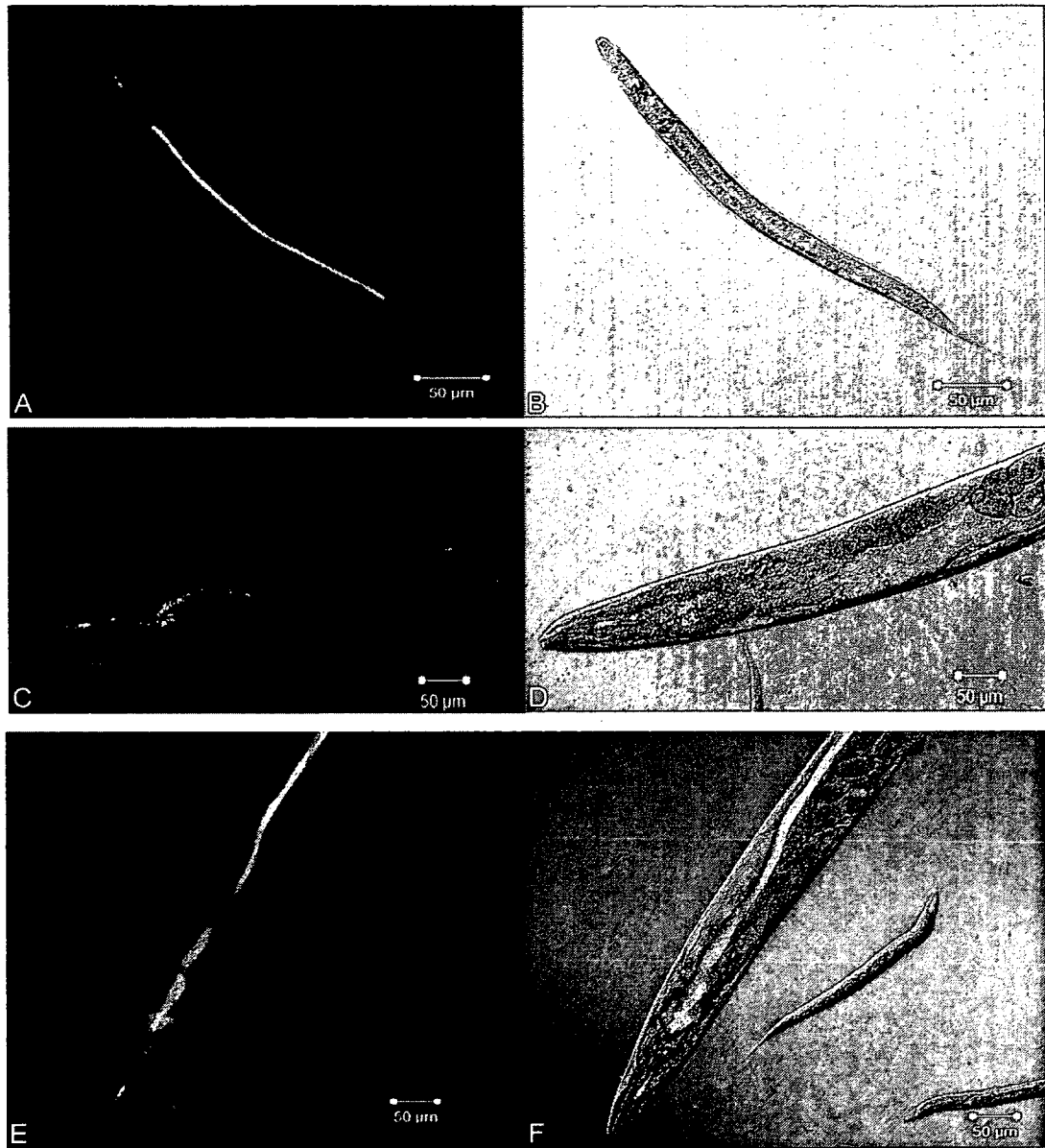


Figure 4.2. Pair-wise competition of *V. parahaemolyticus* strains BB22 and GacA⁻ mutant of BB22 in *C. elegans* with and without norepinephrine. CLSM images of GFP labeled BB22 and RFP labeled GacA⁻ mutants without exposure to norepinephrine (A and B), BB22 and GacA⁻ mutant exposed to norepinephrine (C and D), and BB22 and GacA⁻ both exposed to norepinephrine (E and F).

Chapter IV

Significance, Limitations, and Future Directions

Despite extensive preventative measures in place, foodborne diseases are still a constant problem in the United States. The CDC has estimated that there are 48 million cases of foodborne illness with *V. parahaemolyticus* as the leading cause from ingestion of contaminated shellfish. The incidence of *V. parahaemolyticus* infection based on COVIS increased 13-fold or 1200% from 1996 to 2010 whereas according to FOODNET it increased 3.8-fold or 283% (Newton et al 2012). The rise in human incidences may be due to increased contact with pathogenic strains from the environment, which may in turn be due to rising water temperatures from global climate change, promoting proliferation of both environmental harmless and pathogenic *V. parahaemolyticus* (Hanson et al 2006, Newton et al 2012).

The current limiting factor to the proper risk assessment of the emergent pathogen *V. parahaemolyticus* is a lack of knowledge of this organism's virulence characteristics (FDA). Surveillance projects aimed to monitor the levels of *V. parahaemolyticus* and characterizing the strain population are currently conducted in many locations, however, the current virulence markers used to indicate virulence potential are unreliable in distinguishing pathogens from harmless environmental strains. An abundance of strains with *tdh* and *trh* does not always correlate to an outbreak and virulence is not affected in infection models when these genes are knocked out (DePaola et al 2003, Lynch et al

2005). Thus, it is necessary to gain a better understanding of the mechanism of virulence and distinguishing characteristics that would allow the discrimination of pathogens from the harmless environmental strains. The purpose of elucidating virulence associated traits is to distinguish genetic markers that can be utilized to identify the presence of pathogenic strains from the environment. A major obstacle for the identification of these virulence traits is the lack of a high-throughput model that can effectively emulate the human gastro-intestinal tract.

Current models of infection include the murine model, the rabbit ileal loop model, piglets, and cell culture methods (Boutin et al 1979, Caburlotto et al 2010, Calia et al 1975, Hiyoshi et al 2010). The proper use of these models requires extensive expertise and the major limitation is the vast number of animals that would be needed for infection is not feasible. Microbe-host studies have shown that innate defense mechanisms are conserved among plants, invertebrates, and mammals (Starkey et al 2009), which suggests that among diverse lineages there are some universal host infection responses. In a study with *P. aeruginosa*, some of the virulence factors that were necessary to infect mice model were also necessary in plants, nematodes, and insect models (Hendrickson et al 2001). The use of alternative models as surrogates for infection was evaluated with the use of many pathogens, such as *V. cholerae*, *V. vulnificus*, and *S. marcescens* (Blow et al 2005, Cinar et al 2010, Dhakal et al 2006).

From the assessment of multiple alternative models for infection, we found that models such as *D. melanogaster* and *M. sexta* were too variable between replicate experiments which make them unreliable infection models. *C. elegans*, on the other hand, is sensitive to subtle virulence differences among different *V. parahaemolyticus* strains

(Chapter 2). The physiological structure and short life cycle with large amounts of offspring makes this model useful in assessing infections within the gastrointestinal tract as well as providing large numbers of animals for infection. The genetic tractability and availability of immune-compromised mutant strains of *C. elegans* will enable the study of host-microbe interactions during infections.

Similar to previous studies in mice (Mahoney, 2011), the GacA⁻ mutant displayed defective pathogenesis within *C. elegans* (Chapter 2); however, within pair-wise competitions, the GacA⁻ mutant is more fit in nematode intestine based on a competitive index of 2.89 (Chapter 4). In addition, we were able to assess the effect of the human hormone, norepinephrine, on bacterial virulence during infection of a host. Virulence of both pathogenic and benign strains was promoted within nematodes when exposed to the presence of norepinephrine. The presence of this host cue was sufficient to re-establish the virulence of the GacA⁻ mutant to wild-type levels and affect the selective advantage within competitions. Elucidation of the interaction between this hormone and *V. parahaemolyticus* virulence may provide insight on global virulence responses to host cue.

The current limitation with *C. elegans* is the lack of a high-throughput protocol for screening a transposon generated mutant library for altered virulence. The detection of changes in virulence-associated traits and the molecular identification of the associated genes provide no information on their contribution to virulence. As seen from the nematode assay performed on capsule mutants, the alteration of a specific phenotypic trait expression can alter the overall virulence potential of the bacteria (Chapter 3). Our findings have shown that capsule mutants have exhibited a general decrease in virulence

within infected nematodes, which correlates to a study in *V. vulnificus* showing a decreased virulence in mice due to decreased capsule expression (Wright et al 1990). Based on these findings, capsule production contributes to the virulence of *V. parahaemolyticus*.

Without the ability to screen the entire library, genes that can alter virulence without inducing phenotypic changes will not be identified. The current virulence markers are not able to effectively distinguish pathogens from the large population of harmless strains which impedes the ability to predict and prevent future outbreaks. Thus, the development of a high-throughput model sensitive enough to detect small virulence shifts is crucial to identifying important genes within *V. parahaemolyticus*. Previous studies utilized the plant model, *A. thaliana*, at a high-throughput model for identifying *P. aeruginosa* virulence factors (Rahme et al 1999). In a comparative study looking at the virulence genes involved in mice pathogenicity, it was shown that most of these genes were crucial in establishing virulence in *A. thaliana* and *C. elegans* (Hendrickson et al 2001). Thus, *A. thaliana* may be a better alternative in screening *V. parahaemolyticus* library mutants for alterations in pathogenicity. The main concern with the use of a plant model for *V. parahaemolyticus* infection studies is the lack of a gastrointestinal tract, which is the normal route of infection for this pathogen. In order to efficiently screen for mutants altered in virulence and assess microbe-host interactions that emulate human disease, a combination of infection models may be necessary to elucidate the virulence mechanism of this pathogen.

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Appendix I

Solid substrate virulence assay with *C. elegans*

Important Notes:

****Plates for experimental preparation are the round Petri plates**

****Plates for nematode maintenance are the large square plates**

Day 1:

Experiment Preparation

1. Wash L4/Young adult nematodes off the NGM plates with 10.5 ml of M9 buffer.
2. Pipette nematodes in M9 with a p1000 into a 15 ml conical tube.
3. Centrifuge at 1,000 rpm for 7 minutes.
4. Remove supernatant, leaving about 0.3 ml suspension with nematodes, and wash with 5 ml M9 buffer.
5. Centrifuge at 1,000 rpm for 4 minutes.
6. Repeat steps 4 and 5 twice.
7. Remove supernatant and leave about 0.5 ml nematode suspension and add 5 ml of M9 buffer.
8. Pipette 100µl of the nematode suspension onto each assay plate. Make sure to spot close to the edge of the plate and 1 cm away from the bacterial lawn so dead nematodes plated can be differentiated from dead nematodes from infection.
9. Allow the plates to dry at room temperature and then place into the incubator.
10. For 37°C, place into incubator for 5 hours (no more than 7 hours) and then move to 28°C incubator.

Nematode Maintenance

1. Transfer remaining nematodes from the experimental preparation onto new large square NGM plates with E.coli OP50.
2. Inoculate 3 ml of LB with 5 E.coli OP50 colonies and incubate at 37°C.

OR

Nematode Transfer

1. Wash nematodes off the NGM plates with 10.5 ml of M9 buffer
2. Pipette nematodes in M9 with a p1000 into a 15 ml conical tube.
3. Centrifuge at 1,000 rpm for 7 minutes.
4. Remove supernatant, leaving about 0.5 ml suspension with nematodes.
5. Combine nematodes in multiple conical tubes to mix the population.
6. Pipette nematode suspension across the top of the plate.
7. Keep plates on the bench top at room temperature.

Nematode Maintenance

1. Inoculate 3 ml of LB with 5 E.coli OP50 colonies and incubate at 37°C.

Day 2:

Experimental Preparation

1. Score the experimental nematodes for 24 hour results under the dissecting scope.

Nematode Maintenance

2. Spread 500µl of E.coli OP50 onto a large square NGM plate.

3. Incubate large square plate at 37°C.

Day 3:

Experimental Preparation

1. Score the experimental nematodes for 48 hour results under the dissecting scope.

Nematode Maintenance

2. Transfer the nematodes plated on Monday to the new NGM plate with E.coli OP50 lawn.
3. Wash the nematode plate with 10.5 ml of M9 buffer.
4. Pipette nematodes in M9 into a 15 ml conical tube.
5. Centrifuge at 1,000 rpm for 7 minutes.
6. Remove supernatant, leaving about 0.5 ml suspension with nematodes.
7. Combine nematodes in multiple conical tubes to mix the population.
8. Pipette nematode suspension across the top of the plate.
9. Inoculate 3 ml of LB with 5 E.coli colonies and incubate at 37°C.

Day 4:

Experimental Preparation

1. Streak out strains for nematode assay.

Nematode Maintenance

1. Spread 500µl of E.coli OP50 onto a large square NGM plate.
2. Incubate large square plate at 37°C.

Day 5:

Nematode Synchronizing

1. Bleach the nematodes by washing the nematodes off the NGM plates with 10.5 ml of **ICE COLD** M9 buffer.
2. Using a p1000, pipette nematodes in M9 into a 15 ml conical tube (should be ~9ml in each).
3. Add 3 ml of **ICE COLD** bleach solution (2 ml of bleach with 1 ml of 5M NaOH) to the conical tube.
4. Incubate tubes on ice for 10 minutes and shake the tubes every 2 minutes for 10 seconds (**KEEP ON ICE!!**).
5. Centrifuge at 1,000 rpm for 7 minutes at room temperature.
6. Remove the supernatant and resuspend the nematode pellet in 2 ml of **ICE COLD** M9 buffer.
7. Vortex briefly and centrifuge at 1,000 rpm for 3 minutes at room temperature.
8. Remove supernatant and resuspend the nematode pellet in 1 ml of **ICE COLD** M9 buffer.
9. Vortex briefly and centrifuge at 1,000 rpm for 2 minutes at room temperature.
10. Remove supernatant leaving around 300 µl and plate the remaining liquid along the bacterial lawn of a new NGM plate seeded with E.coli OP50 from day 4 step 2.
11. Leave plates on bench top at room temperature.

Day 6:

Experimental Preparation

1. Start 5 ml broth cultures of experimental strains and grow at optimal growth conditions for strains.

Day 7:

Experimental Preparation

1. Spread 100µl of each bacterial strain onto a separate NGM petri plate (6 plates per strain).
2. Incubate 3 of the plates at 37°C and the other 3 plates at 28°C overnight.

Nematode Maintenance

1. Spread 500µl of *E.coli* OP50 culture onto large square NGM plate.
2. Incubate square NGM plate at 37°C.

To Start Nematodes From Freezer Stock:

1. Scrap a small chunk of the frozen stock and place it in the center of a large NGM plate that has an overnight *E.coli* OP50 lawn.
2. Keep the plate on the bench top and check for nematode movement after 3 days and up to 1 week. If no movement after 1 week, then try again.

****Frozen nematode stocks are labeled N2-CGC in shelf 3, metal rack 3 of the old -80°C freezer**

****Only ~30-40% chance of revival/recovery of the nematodes from freezer stock**

Protocol was modified from Cooper et al, Hendrickson et al, Sellegounder et al, and Stiernagle et al.

Scoring Protocol:

1. Set the objective lens on the dissecting scope to 1.5.
2. Place the assay plate upside down and allow nematodes to acclimate to the light intensity for 1 minute.
3. Scan the entire plate to get an idea of the general rate of movement to count 15 nematodes per treatment that represent the mobility rate of the general population.
4. Use a stop watch to count the amount of time it takes for infected nematode to move 2 cm using the ruler on the lens.

****Avoid counting nematodes outside the bacterial lawn****

Tips for handling nematodes:

****Keep an old nematode plate on top of the newest ones to prevent contamination from occurring too often**

****Do not keep nematode stocks in the 28°C incubator, nematodes will live but stop procreating after a few weeks!!!**

****Nematodes can only survive at 37°C for up to ~7 hours**

Media Recipe:

Nematode Growth Media (500 ml):

Sodium chloride	1.5 g
Bacto-peptone	1.25 g
Agar	10 g
After Autoclaving	
Potassium phosphate buffer, pH 6.0	12.5 ml
1 M Magnesium sulfate heptahydrate	0.5 ml
1 M Calcium chloride	0.5 ml
5 mg/ml Cholesterol	0.5 ml

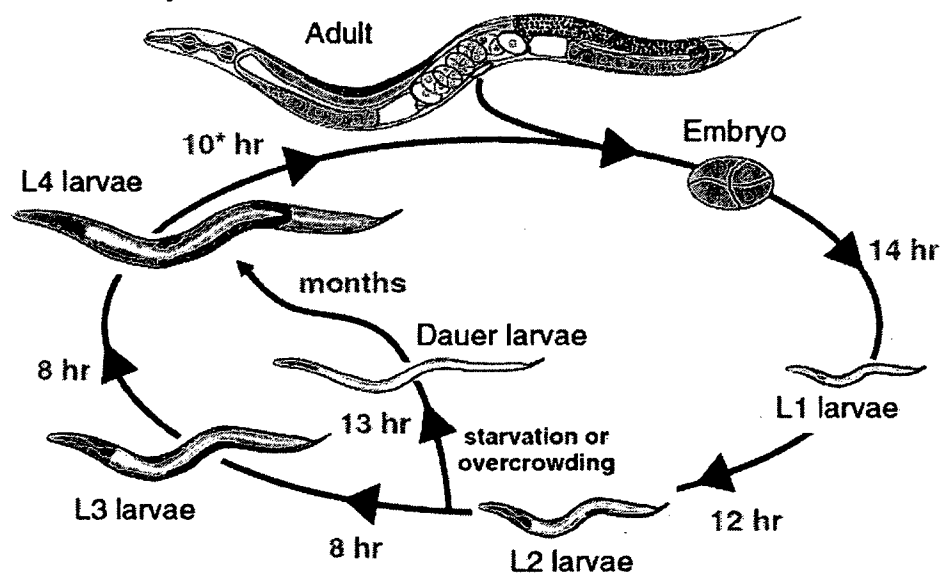
M9 bufferr (500 ml):

Potassium phosphate dibasic	1.5 g
Disodium phosphate	3.0 g
Sodium chloride	2.5 g
After Autoclaving	
1 M agnesium sulfate heptahydrate	1.0 ml

Bleach Solution (9 ml):

5 M Sodium hydroxide	3.0 ml
Household bleach	6.0 ml

Nematode Life Cycle:



Baylis et al.